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The sheath investment of Gloeothece ATCC 27152 : its chemical characterization and heavy metal binding ability.

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THE SHEATH INVESTMENT OF *GLOEOTHECE* ATCC 27152. ITS CHEMICAL CHARACTERIZATION AND HEAVY METAL BINDING ABILITY

A Dissertation Presented

by

Bruce Edward Tease

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To my

Parents

and Brother

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ABSTRACT

THE SHEATH INVESTMENT OF GLOEOTHECE ATCC 27152
ITS CHEMICAL CHARACTERIZATION
AND HEAVY METAL BINDING
ABILITY

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A procedure was developed to remove the sheath investment of the chroococcacean cyanobacterium Gloeothece ATCC 27152. Chemical analyses and heavy metal binding studies, with cadmium, were performed on isolated sheath investments. The neutral sugars; rhamnose, 2-0 methyl xylose, xylose, mannose, galactose and glucose, together with the uronic acid; mannuronic, glucuronic and galacturonic acid, constituted the acidic heteropolysaccharide comprising the sheath investment material. Protein, pyruvic acid, o-acetyl and sulfate groups were also components of the sheath investment of Gloeothece ATCC 27152. The maximum pore size of the sheath investment was estimated to be approximately 16 n m in diameter. The sheath investment composition and physical appearance changed when cells of Gloeothece ATCC 27152 were grown under nitrogen fixing conditions Consequently, the metal binding capacity varied between the sheath investment preparations. The extent and significance of these alterations are discussed.

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CHAPTER ONE

INTRODUCTION

In nature, most cyanobacteria, like other bacteria, produce gelatinous extracellular material, primarily composed of acidic polysaccharides. Polymers of sugar molecules interact to form hydrated capsules or amorphous shrouds that surround the cell (or groups of cells), providing protection against dessication and phagocytic predation, as well as, assisting in attachment and nutrient uptake.

Glycocalyces and slime layers occur in both groups of procaryotes but the structural diversity of cyanobacterial extracellular layers is greater. Certain cyanobacteria are capable of producing a 'sheath investment' (a very resilient, well delineated, sac-like envelope) whose complexity is at least structurally and perhaps chemically unique.

How this layer differs from the glycocalyx and slime layer remains uncertain. Determination of the chemical composition of sheath investments would be the first step towards understanding the complexity of these external layers. Their function in the physiology and

ecology of cyanobacteria could then be more easily understood.

The information presently available on cyanobacterial external layers, in general, is meager. Further-more, inconsistencies have emerged in external layer terminology that injects an element of uncertainty in research performed to date. Capsule, glycocalyx, sheath, mucilage, slime, integument, and investment represent terms which have been used interchangeably to describe these layers. Of these terms, sheath has been used most liberally, describing virtually every type of external layer. Several authors have attempted to remedy the situation but incongruities remain (Martin & Wyatt, 1974; Stanier & Cohen-Bazire, 1977; Vaara, 1982 and Drews & Weckesser, 1982).

In this study, extracellular layers are grouped into three categories. Type I: Slime or mucilage is considered as an amorphous, gelatinous matrix that exudes into the surrounding medium. Type II: Capsule or glycocalyx refer to a relatively insoluble layer of extracellular material, adherent to the cell envelope but usually possessing a more diffuse outer boundary. Type III: Sheath investments represent resilient

structures that need not adhere to the cell envelope possessing a distinct outer boundary.

The sheath investment form of external layer is mainly observed in unicellular cyanobacteria of the generic classification scheme, section I sub-group (Rippka et al., 1979). In cells of Microcystis, Chroococcus, Gloeocapsa, Gloeothece and Gloeobacter, a sheath investment initially forms adjacent to the outer membrane. Following cell division, the sheath investment detaches from the outer membrane and expands to accomodate the growing population that it encloses. Except for Microcystis, the individual cells produce new layers following cell division. This results in several ensheathed clusters of 2-4 cells, constituting a colony containing 4-16 cells (Stanier & Cohen-Bazire, 1977 and Kallas et al., 1983).

The intent of this study was to determine the chemical composition of sheath investments collected from cultures of the chroococcacean cyanobacterium Gloeothece ATCC 27152, grown with and without (fixing atmospheric nitrogen) a combined nitrogen source ($NaNO_3$). The ability of this organism to fix atmospheric nitrogen (Wyatt & Silvey, 1969; Gallon et al., 1972 and Kallas et al., 1983) was exploited in

determining the influence of environmental conditions on the composition of sheath investments. The alteration of a few strategical constituents (i.e. uronic acids) could induce major physical changes in sheath investment conformation. Consequently, the diffusion of various gases and nutrient ions could then be notably affected. The presence and significance of such changes in the sheath investment of Gloeothece ATCC 27152 is presented.

CHAPTER TWO

LITERATURE REVIEW

2.1 Functional Aspects of External Layers

External layers occupy a strategic position at the outermost region of the cell envelope and consequently play a fundamental role in the interaction of cells with their environment. Although a specific assessment of the functional aspects of sheath investments is lacking, a number of benefits have been attributed to the glycocalyx and slime layer of bacteria and cyanobacteria that may have some relevance to sheath investments.

It has been accepted for quite some time, that the chance of survival for many organisms is improved by thigmotropism (Zobell, 1943). Once in contact with a substrate, external layers serve to anchor the cells, thereby, permitting its occupation by additional organisms (Daniels, 1980). In a similar way, external layers modify the viscosity and flow pattern of the

water in their immediate vicinity. The sinking rate of planktonic organisms is reduced and as a result they are maintained at favorable light levels for longer periods of time (Fogg, 1971). Since the mucilage of cyanobacteria generally has a density less than that of the cell and closer to that of water, buoancy is, consequently, improved (Wetzel, 1975).

The active metabolism of surface adhering bacteria was attributed to the gelatinous films they produce (Zobell & Anderson, 1936 and Zobell, 1943). Lange (1976) suggested that the external layers of cyanobacteria sequester essential nutrients that are present at submarginal concentrations. The greater availability of trace elements at the cell surface enhances their uptake into the cell. It is possible, then, that external layers enhance cell metabolism by facilitating nutrient uptake in an oligotrophic environment.

External layers have been implicated in reducing the rate of diffusion of certain gases to and from the cell (Paerl, 1978; Chang, 1980 and Geesey, 1982). The external layer of Oscillatoria rubescens was found to reduce the rate of carbon dioxide uptake by 30% in laboratory grown cultures (Chang, 1980). In a similar fashion, external layers are thought to facilitate

nitrogen fixation in oxygen saturated environments (Paerl, 1978). The sheath investment of Gloeothece ATCC 27152 was shown to be a stable feature of cultures grown with and without a combined nitrogen source (Kallas et al, 1983). Grover and Puri (1978) suggested that the sheath investment induced favorable conditions, sufficiently microaerophilic to permit nitrogen fixation. Kallas et al (1983), however, concluded that the sheath investment had no role in protecting the nitrogenase system from oxygen inactivation, based on work with sheathless wild type and mutant cells of Gloeothece ATCC 27152. Cultures of Aphanothece (Synechococcus) were, likewise, shown to aerobically fix atmospheric nitrogen, despite the inherent genetic inability to produce sheath investments (Singh, 1973). Rather than the physical exclusion of oxygen as the mechanism preventing nitrogenase inactivation, the sheath investment may serve to aggregate cells into dense clusters, in which photosynthesis is excluded and respiration serves to lower the oxygen tension. Cells within the aggregates are differentiated in regards to their spatial location with-in the colony, rather than by physical alteration in cell structure (i.e. heterocysts) Rodrigues (1986,

personal communication) noticed excessive clumping of the sheathless mutant of Gloeothece ATCC 27152. Such a growth mode could enhance oxygen exclusion and therby permit the fixation of atmospheric nitrogen. Future investigations are required in order to clarify the role sheath investments play in facilitating nitrogen fixation.

External layers play a vital role in the symbiotic association between cyanobacteria and bacteria (Bershova et al., 1968; Kuentzel, 1969; Kessel & Eloff, 1975; Overbeck, 1975 and Caldwell & Caldwell, 1978). A model for attachment of bacteria to cell surfaces of other organisms has been presented by Costerton et al (1978). Anionic groups with in the acidic polysaccharide of the bacterial glycocalyx cross-link with the extracellular polysaccharide of the cyanobacterium (or a facsimile) by forming polar bonds, mediated by divalent cations in the medium. Consequently, a stable association is established without a direct cell to cell contact that could alter what is otherwise a symbiotic relationship. Components of external layers as well as cell exudates are often utilized by various heterotrophic bacteria which provide carbon dioxide and consume oxygen, thereby,

stimulating photosynthesis and atmospheric nitrogen fixation, respectively (Engelman, 1881; Lange, 1967; Caldwell & Caldwell, 1978; Chrost & Brzeska, 1978; Herbst & Overbeck, 1975; Paerl & Kellar, 1978 and Paerl, 1979). Steep chemical gradients, formed within 10 uM of the cell surface, create a microenvironment where concentratons of various substances vary considerably from those found in the surrounding milieu. Using tetrazolium salts, Paerl & Bland (1982) have shown that microbes lower the oxygen tension in these microzones to subsaturated, microaerophilic and even to anaerobic levels in waters previously supersaturated.

Theoretically, external layers provide a suitable environment for efficient operation of extracellular enzymes (Tonn & Gander, 1979). Although protein has been detected in the sheath investment of several cyanobacteria (Schrader et al, 1982; Anemiya & Nakayama, 1984 and Jurgens & Weckesser, 1985) further research is required before a structural or enzymatic function can be inferred.

Several authors have reported that cyanobacteria have significant binding capacities (Harvey & Patrick, 1967; Gibson, 1972; Gadd & Griffiths, 1978; Van Den Berg et al., 1979; McKnight & Morrel, 1979; Laube et al., 1980; Allen et al., 1980; Crist et al., 1981; Les & Walker, 1983 and Anemiya & Nakayama, 1984), but the relative contribution of the external layers to this adsorption has not been addressed. Anemiya and Nakayama (1984), working with Microcystis, have shown that sheath investments contained substantially more metal ions than whole cells. Binding affinities of the sheath material exhibited the following sequence: Fe>Zn>Mn>Cu>Ni.

Although the affinity of external layers (particularly slime layers) for heavy metals may provide some degree of protection against heavy metal toxicity (Fazio et al., 1982 and Dudman, 1977) bioaccumulation and passage of the heavy metals through the food chain may be a more devastating consequence (Patrick & Loutit, 1976). Cyanobacterial external layers are considered to contribute significantly to the organic colloid content of natural waters (Leppard et al., 1977). Coupled with their affinity for metal ions and the increase in contamination of aquatic

systems by heavy metals, the interaction of heavy metals with external layer material should be of major environmental concern in the study of lake dynamics.

Other less studied benefits imparted by external layers include protection against lysis by bacteria and viruses and protection against dessication. Studies with myxobacter have shown that cell lysis only occurred when there was a direct contact between the polar ends of the myxobacter and the cyanobacterium (Shilo, 1970). By preventing access to the cell surface, external layers may serve a vital role in population dynamics by sustaining populations that would normally be destroyed by the lytic action of certain bacteria and viruses. The hygroscopic nature of external layers would provide a natural resistance against dessication for organisms temporarily exposed to atmospheric conditions (Geesey, 1982). In both cases, however, little experimental evidence is available to support these claims.

2.2 The Effect of Changes in Environmental Conditions on External Layers

Changes in culture conditions were shown to affect the quantity of external layers (primarily capsules and slime layers) produced by bacteria and cyanobacteria. Increased production of extracellular polysaccharides appeared to be directly proportional to the C:N ratio. Carbon utilization was directed to extracellular polysaccharide production, rather than to the production of intracellular material, when nitrogen was limiting or present in a less readily metabolized form (Sangar & Dugan, 1972 and Tischer & Davis, 1971). The quantity of external layer material produced was also dependent upon light levels, temperature, culture age and sulfur, phophorus and potassium concentrations (Duguid & Wilkinson, 1953; Wilkinson et al., 1954; Moore & Tischer, 1964; Findley et al., 1970; Sangar & Dugan, 1972; Mehta & Vaidya, 1978 and Konopka & Schnur, 1980). While changes in culture conditions were shown to have an effect on the quantity of external layer material produced, there have been no studies on the effect of these changes on individual components of cyanobacterial external layers.

Changes in the primary structure of polysacharides are known to greatly affect their physical properties (Rees, 1975; Rees, 1977 and Morris et al., 1980). Addition or deletion of a few residues could change the porosity and metal binding capacity of external layers in such a way as to significantly affect the physiology of the organism, with positive as well as negative connotations.

Structural modification of the glycocalyx of pathogenic bacteria has been suggested as a means of maintaining pathogenicity by avoiding antibody recognition (Troy, 1979 and Smith, 1977). Changes in the physical properties of external layers would also accompany similar modifications. Pyruvic acid and O-acetyl groups are common constituents of bacterial external layers and their presence or absence is thought to greatly affect the physical properties of these structures (Sloeneker & Jeanes, 1962). Uronic acid residues, responsible for the anionic nature of acidic polysaccharides (Rees, 1975), significantly contribute to the metal binding ability of this material (Ikeda et al., 1982). Alteration in these residues have been shown to affect the ion exchange properties of alginic acid (polymannuronic-guluronic

acid) extracted from several species of brown algae as well as the extracellular alginate isolated from the bacterium Azotobacter vinelandii (Haug & Larsen, 1971). Calcium ions mediate cross-linking of the polysaccharide chains and thereby create stiffer gels in material possessing a greater affinity for calcium ions. Younger cells contain a predominance of guluronic acid, which possesses a greater binding affinity for calcium ions than mannuronic acid. Consequently, the physical properties of the alginate gels vary with the age of the cell, becoming stiffer as the cells grow older.

It was observed that the production of the slime layers of Achromobacter sp. was stimulated in the presence of high concentrations of heavy metals (Zevenhuizen & Ebbink, 1974). It remains uncertain whether this is a direct response to environmental stimuli or simply a coincidental occurence. The ability of extracellular polysaccharides to bind metals would lead one to speculate this to be a detoxifying mechanism that might be regulatable by the cell (Zevenhuizen & Ebbink, 1974).

The composition of the glycocalyx of Pseudomonas atlantica was found to change during its growth cycle.

Significant increases in both the proportion as well as in the absolute amounts of glucuronic and galacturonic acids were observed late in the stationary phase, a point in the growth cycle when cells showed maximal physiological stress (Unlinger & White, 1983). Changes in the composition of sheath investments would appear to be plausible. However, there has been no research performed to date regarding this possibility.

2.3 Chemical Composition of External Layers

The functional aspect of any structure is dependent upon the type and arrangement of its individual components. The composition and sequence of amino acids determines the conformation and consequently the biological function of a particular protein molecule. An understanding of the chemical components of external layers would, likewise, be essential in assessing their role in cellular functions. Unfortunately, the chemical analysis of external layers is arduous and the task is often complicated by the inability to separate and/or isolate the material from cellular components. Often what is analyzed represents only a fraction of the whole. Assuming the entire structure is obtainable, an additional problem occurs regarding complete hydrolysis of the material without incurring breakdown of less resistant components.

The complexity of external layers was recognized by Klein (1915) who attributed their resiliency to certain pectinaceous components. Hough et al., (1952) while investigating the polysaccharide content of various freshwater algae, commented on the problems associated with the chemical analysis of the slime layer of Nostoc

sp.: "The purification of the mucilage was difficult and tedious, and the hydrolysis products so complex as to render analysis difficult.". The heterogeneity of this material and its stability to acid hydrolysis made it impossible to confidently report the relative proportions of the components detected.

While external layers are no longer considered to be composed of pectin (polygalacturonic acid) staining by the anion-selective dyes, ruthenium red and alcian blue, is indicative of the presence of uronic acid residues (Pate & Ordal, 1967). Despite the availability of several analytical methods for the determination of uronic acids (Jones & Albersheim, 1972; Bociek & Welti, 1975; Vadas et al., 1981 and Fazio et al., 1982), their quantitative as well as qualitative analysis is very difficult. The carboxyl group of uronic acids stabilizes the glycosidic linkage of the polysaccharide to acid hydrolysis (Lindberg et al., 1975). Neutral sugar residues are either destroyed, due to the necessity of prolonged acid hydrolysis and/or interfere with analytical procedures. Until procedures are developed that address the resiliency of the carboxyl group towards acid hydrolysis, research reporting uronic acid content should be considered, at best, only

approximations.

Chemical analyses have revealed that the glycocalyces and slime layers of several cyanobacteria contain a variety of neutral sugars and uronic acids (Table 1) (Hough et al., 1952; Bishop et al., 1954; Biswas, 1957; Moore & Tischer, 1965; Dunn & Wolk, 1970; Kokyrsta & Chekoi, 1972; Sangar & Dugan, 1972; Wang & Tischer, 1973; Mehta & Vaidya, 1978 and Painter, 1983). Deviation in sugar content between similar species have been attributed to utilization of separate strains and/or variations in culture conditions. However, differences in isolation and analytical procedures seem more plausible (Tischer & Moore, 1964 and Mehta & Vaidya, 1978).

The chemical characterization of the external layer of Chlorogloeopsis PCC 6912 was the first work performed on the more structurally complex sheath investment of cyanobacteria (Schrader et al., 1982). Isolation and purification of this material was facilitated by its high protein content (22%) which gave the molecule a density greater than that of whole cells. Homogenization of cultures liberated sheath investments that maintained their shape throughout the isolation procedure. Low speed centrifugation was used

to pellet intact sheath investments from less dense cellular debris. An acidic heteropolysaccharide containing glucose, mannose, galactose, arabinose, xylose, an unknown sugar, traces of rhamnose and fucose and glucuronic acid represented the carbohydrate portion of the sheath investment.

The sheath investment of Microcystis was isolated by continuous centrifugation, followed by filtration and lyophilization. This material was shown to contain 35-47% polysaccharide, which contained uronic acids, and 18-24% protein (Anemiya & Nakayama, 1984).

Recently, chemical analyses were performed on the cell envelope of two chroococcacean cyanobacteria (Jurgens & Weckesser, 1985). The sheath investment of Gloeothece PCC 6501 contained 4% protein and the neutral sugars were: glucose, galactose, mannose, 2-O-methylxylose and rhamnose. The polysaccharide constituents of the sheath investment of Chroococcus minutus were dominated by the O-methyl sugars; 2-O-methyl-6-deoxyhexose, 2-O-methyl-dideoxyhexose, 2-O-methylhexose and 3-O-methylhexose. Protein accounted for 6% of the sheath investment.

The work of Schrader et al.(1982) represents the only comprehensive study presented thus far on sheath

investments of cyanobacteria. Jurgens & Weckesser (1985) were concerned mainly with the cell wall fractions and only briefly addressed the chemical composition of sheath investments. Similarly, Anemiya & Nakayama (1984) concentrated on the binding capacity of material isolated from the sheath investments of Microcystis and did not elaborate on the chemical nature of this singular colonial external layer. The heteromorphic cultures of Chlorogloeopsis PCC 6912 were, unfortunately, not in synchrony, resulting in the growth of several cell types: heterocystic trichomes, single cells, multicellular clusters and akinetes. The sheath fraction obtained, consequently, could not be assigned to any one cell type nor could the components identified be considered constituents of the same sheath investment.

Protein appears to be absent in the glycocalyx and slime layers studied thus far, while every sheath investment analyzed contained from 4 to 24%. Attempts to remove this component with lysozyme and SDS were unsuccessful. Further research is necessary, however, before the nature of the protein can be determined.

2.4 Ultrastructure of External Layers

The ultrastructure of external layers have been studied for a number of cyanobacteria (Leak, 1967; Tuffery, 1969; Lamont, 1969; Findley et al., 1970; Butler & Allsopp, 1972; Kessel & Eloff, 1975; Schrader et al., 1982 and Vaara, 1982). Like the glycocalyx of bacteria, however, cyanobacterial external layers are difficult to observe in the electron microscope, due to their innately low density and the deformation that occurs during fixation procedures (Roth, 1977). The use of specific antibodies have helped stabilize bacterial external layers against condensation during dehydration procedures but this often results in masking the fine detail of these structures (Bayer et al., 1985). A new method was recently developed that utilizes dimethyl formamide for chemical dehydration of encapsulated bacteria. The progressive lowering of the temperature during dehydration, combined with gelatin enrobement of fixed cells, preserves the external layer conformation without the use of antibodies. Thin fibers, 2-4 nm in diameter were observed, extending radially through the entire capsular area (Bayer et al., 1985).

None of the ultrastructural studies performed thus

far on cyanobacteria have accounted for the drastic condensation of external layers accompanying conventional fixation procedures. The recent advances in electron microscopy of bacterial capsules have allowed the visualization of these structures in their most natural conformation and it now appears that all bacteria, isolated from various ecosystems, are surrounded by external layers (Costerton et al., 1985). Until similar precautions are followed, the true nature of these structures, among cyanobacteria, will continue to be missunderstood.

2.5 Strain History of Gloeothece ATCC 27152

The history of cyanobacterial systematics has been a chronology of alteration and confliction. The criteria for classification, including the arrangement of sub-groups in relation to each other, has been greatly transposed during the past century by many authors: Kirschner, 1898; Fremy, 1934; Elenkin, 1936; Geitler, 1942; Fritsch, 1945; Drouet, 1951; Drouet & Daily, 1956; Desikachery, 1959; Stanier et al., 1971; Rippka et al., 1979; Griffiths, 1984 and Rippka & Herdman, 1985. Papenfuss (1955) attributed this disparity of opinion to the paucity of well-defined characters and the existence of intermediate forms that prevent the establishment of clear cut taxonomical groups.

The number of revisions that have occured in the nomenclature and classification of Gloeothece ATCC 27152, during the past fifteen years exemplifies the confusion that has and may still exist in cyanobacterial systematics. Changes in nomenclature occurred during the passage of cultures from one collection to another, with each group assigning their own designation (Stanier et al., 1971; Gallon et al., 1975; Rippka et al., 1979 and Gallon, 1980). The

erroneous assignment of Gloeothece to the genus Gloeocapsa (Allen & Stanier, 1968) additionally contributed to the confusion. Gloeothece ATCC 27152 appears in the literature as: Gloeocapsa IUCC LB 795 - Indiana University Culture Collection (Wyatt & Silvey, 1969), Gloeocapsa UTEX LB 795 - Culture Collection of Algae at the University of Texas (Gallon, 1980), Gloeocapsa CCAP 1430/3 - Culture Collection of Algae and Protozoa, Cambridge, England (Gallon, 1980) and Gloeothece PCC 6909 - Pasteur Culture Collection (Rippka et al., 1979). Gloeothece ATCC 27152 and Gloeothece ATCC 27151, that were isolated in 1926 (Starr, 1964) and 1965 (Allen & Stanier, 1968), respectively, possess nearly identical DNA base compositions and are considered to be independent isolates of the same organism (Rippka et al., 1971 and Stanier et al., 1971).

Most recently, the phycological classification system was superseded by generic descriptions based upon structural and physiological properties of axenically grown laboratory cultures (Stanier et al., 1971; Stanier & Cohen-Bazire, 1977 and Rippka et al., 1985). Presently, five major sub-groups of cyanobacteria are recognized (Table 2).

The model organism in this study, Gloeothece ATCC 27152 is a member of the section I sub-group, which includes spherical, cylindrical or oval shaped unicells, that reproduce by binary fission, or by budding. Division in one to three planes and the presence or absence of thylakoids and sheath investments are major classification traits. Mean DNA base composition (mol% GC) and general metabolic characteristics are used to further delineate strain types. Originally observed by Naegeli (1849), cells of Gloeothece occur in various aquatic as well as terrestrial environments, including wet rocks, mosses, limestone caves and bog pools (West & Fritsch, 1927; Smith, 1950; Waterbury, 1971 and Rippka, 1972).

Specifically, the oval shaped cells of Gloeothece ATCC 27152 (Figure A), divide in one plane and possess well defined multilaminated sheath investments that envelope individual cells as well as small 2-4 celled clusters forming larger aggregates of 4-16 cells (Kallas et al., 1983). Despite being obligate photoautotrophs, these organisms are light sensitive and require light levels of 500-1000 lux or less for optimal pigmentation (Rippka et al., 1971; Stanier et al., 1971 and Kallas et al., 1983) Growth is

consequently slow. Generation times range from 40 to 100 hours, depending upon the particular culture conditions employed (Rippka et al., 1971; Gallon et al., 1972; Millineaux et al., 1981 and Kallas et al., 1983). In addition to chlorophyll-a and carotenoids, the phycobilins, phycoerythrin and phycocyanin also occur, giving healthy cultures their blue-green coloration (Stanier et al., 1971 and Buckley & Houghton (1976).

CHAPTER THREE

METHODS

3.1 Culture Conditions

Gloeothece ATCC 27152 was obtained as a slant culture from the American Type Culture Collection, Rockville, Maryland. Cultivation of healthy cultures necessitated low light intensity and relatively dense inoculums (50ug/ml dry cell weight or approximately 5×10^5 cells/ml). Slant cultures were initially transferred to 5ml glass tubes and loosely covered with screw caps. Each tube contained 1ml of medium BG-11 (Table 3), a modification, proposed by Allen (1968) of medium G-11 (Hughes et al., 1958). Tube cultures were placed in test tube racks and statically grown under continuous illumination of 50 ft-c (1.7 watts/m^2 from a cool white fluorescent light) for 2 weeks at 26 C. Culture biomass was increased by continually transferring exponentially growing cells to larger volumes of sterile BG-11 media. Tube cultures were added to 30ml of medium BG-11 and incubated for 2 weeks at 26 C on a New Brunswick gyrotary shaker (115 rotations/minute). Two cool white fluoresecent lights

provided continuous illumination of 100 ft-c (3.4 watts/m^2). Cells were allowed to settle and were then removed by pipet and aseptically transferred to 50 ml of fresh BG-11 media. Similar transfers were made into 100ml and finally 500ml of fresh media. To permit sufficient diffusion of gases, the combined volume of cells and fresh media did not exceed 20 % of the total container volume (Drew, 1981).

A growth rate study was performed to determine the generation time of Gloeothece ATCC 27152, grown under various culture conditions. Nine, 300ml side arm flasks, containing, in triplicate, medium BG-11, BG-11 minus $NaNO_3$ and BG-11 supplemented with 100mg/L of Na_2HCO_3, were aseptically inoculated with 1ml of a concentrated culture, originally grown on medium BG-11 or BG-11 minus $NaNO_3$. The initial cell density of each flask was approximately 50 ug/ml. The nine flasks including 2 blanks (for spectophotometer zeroing) were placed on the gyrotary shaker, at 26 C under the continuous illumination (100 ft-c or 3.4 watts/m^2) of 2 cool white fluorescent lights. The optical density (OD) was measured daily for 16 days at 450nm. Average generation times were detected by using the following equations:

$$\frac{\log OD_1/OD_2}{.301} = \text{Generations}$$

OD_1= optical density of culture at the start of exponential growth

OD_2= optical density of culture at the end of exponential growth

$$\frac{\text{days between } OD_1 \text{ \& } OD_2}{\text{Generations}} = \text{Generation Time}$$

Sheath removal procedures required a large amount of cells to obtain even small amounts of sheath material. To meet this biomass requirement, exponentially growing stock cultures (500ml) were aseptically transferred to 20 liter glass carboys containing 15 liters of sterile BG-11 media. Prefiltered compressed air was used for aeration and suspension of the carboy cultures. The temperature and light regime were similar for both agitated and aerated cultures.

ACS reagent grade chemicals and deionized-distilled water, obtained from a Millipore Milli-Q water purification system, were used in preparing the culture media. Only borosilicate glassware was used, which after being thoroughly scrubbed, was soaked in 50 % HNO_3 and rinsed several times with

deionized-distilled water. Sterilization at 121 C, under 15 psi, was achieved by autoclaving media in individual culture vessels capped with pre-formed foam plugs and covered with aluminum foil. All culture transfers were aseptically performed in a laminar flow, bacteriological hood. Bacterial and fungal contamination was monitored via microscopic observation using a Zeiss, model IM-35 inverted microscope, equipped with Nomarski illumination (differential interference contrast). Contaminated cultures were discarded.

3.2 Sheath Investment Isolation

The removal and subsequent isolation of the sheath investment of Gloeothece ATCC 27152 was difficult and laborious. A variety of techniques to remove and separate sheath investments from whole cells were attempted but most proved to be ineffective. Centrifugation, disruption by use of: a French pressure cell, Mickle shaker, rotary blender, mortar and pestle and sonicator either yielded no sheath material or were too destructive to the sheath investment and caused excessive cell breakage. A rotary ball mill provided the greatest yield of sheath material with the least amount of cell destruction. The gentle rolling of glass beads over a concentrated cell suspension, separated cells from their sheath investment in a manner analogous to pressing seeds through the skin of a grape (Figure B).

Exponentially growing cells possessed a sheath to cell volume ratio that was most favorable for ball milling. Ensheathed aggregates, containing 4 to 8 cells were dispersed into smaller clusters of 2 to 4 cells after the primary outer sheath investment was ruptured. Attempts to remove the sheath investment of larger aggregates or additional ball milling of the dispersed

clusters, resulted in excessive cell damage.

A density of 10^7 cells/ml was obtained after settling each 15 liter carboy culture and siphoning off the clear media. From a total cell concentrate of 1-2 liters, 30 ml portions were transferred and sealed in a 2 liter porcelin jar containing 250 glass beads, 3 mm in diameter. The jar was revolved for 30 minutes at 4 C. The milled cell concentrate was removed by pipet and the jar was washed with 80 ml of deionized-distilled water. The cell concentrate, plus washings were pooled and evenly distributed among four 30 ml centrifuge tubes.

The crude sheath preparation, in addition to the sheathed aggregates, also contained sheathless unicells and cell debris, including thylakoid membranes and water soluble pigments. Isolation of the sheath material from these components was achieved via differential centrifugation in two stages. The milled cell concentrate was first centrifuged at 250 g for 5 minutes to separate the liberated sheath investments from the denser sheathed aggregates and naked unicells. The supernatant, containing soluble material and cell debris, in addition to sheath investments, was further centrifuged at 1000 g for 5 minutes to sediment the

sheath investments. The resulting supernatant and the pellet from the initial centrifuging were discarded. The viscous sheath pellet subsequently received several deionized-distilled water rinses, each followed by centrifugation at 1000 g for 5 minutes.

The sheath pellet was then resuspended in deionized-distilled water and centrifuged at high speeds (38,000 g) for 10 minutes. The pellet of sheath investments was translucent and green in color. Most cells and debris, not removed via low speed centrifugation, sedimented at the higher speeds beneath the sheath gel pellet. The darker green pellet of cells and debris was removed with a spatula and the sheath gel was resuspended in deionized-distilled water. The above process was repeated until the material from the dark green pellet could no longer be separated from the sheath gel.

This process, although quite effective in separating sheath investments from unicells and sheathed aggregates, was not 100 % efficient. Gradient centrifugation using sucrose and polyethyleneglycol (PEG) was impractical due to the large volume of sheath material handled and the difficulty involved in recovering the sheath investments from the viscous

sucrose and PEG solutions. Ultra centrifugation of the sheath material after the final high speed stage of centrifugation, likewise did not separate the remaining cells and debris from the liberated sheath investments. Complete removal of sheathed aggregates, naked unicells and pigment containing membranes, remaining in the sheath pellet, required treatment with lysozyme, followed by sodium dodecyl sulfate (SDS).

Approximately 17g of the sheath gel was suspended in 30ml of a 1% solution of lysozyme in order to fragment the cell wall of contaminating cell aggregates. The lysozyme interacted with the translucent, green sheath gel and produced a milky, lime green suspension. The pH of the suspension dropped from 7 to 6 as the lysozyme apparently displaced hydrogen ions bound to the sheath gel. The activity of lysozyme was satisfactory at this pH. (Avoiding the use of a buffer eliminated the potential for introducing material difficult to separate from the sheath gel.) The suspension was stirred in centrifuge tubes for 20 hrs, at 4 C. The sheath gel, at the end of lysozyme treatment was centrifuged for 10 minutes at 38,000 g. The interaction with lysozyme caused the sheath gel to dehydrate, dissipating the gel-like characteristic. The sheath

pellet rehydrated after 5 rinses with deionized-distilled water, each followed by centrifugation at 38,000 g. The milky, lime green coloration, however, remained.

A 3% solution of sodium dodecyl sulfate (SDS) was added to each tube of sheath material and stirred for 2 hours at 25 C. The milky, lime green suspension clarified immediately upon addition of the SDS. The sheath gel was centrifuged and washed 10 times with deionized water. A dark green pellet of cell debris collected at the base of the centrifuge tube. The dark green pellet was cell free and, based on its coloration, probably contained thylakoid membranes. Removal of this material from the sheath gel pellet was achieved with a spatula after the clear, green supernatant was poured off.

The sheath investment preparation resulting from this treatment appeared as a transparent, colorless gel that contained no cells. Microscopic inspection of the sheath gel, stained with alcian blue, indicated the investments maintained their conformation throughout the isolation procedures. To prevent bacterial degradation, the sheath gel was lyophilized and stored at -20 C.

3.3 Chemical Analyses

3.3.1 Preliminary microanalysis

Lyophilized sheath investments, obtained from cultures grown with and without a combined nitrogen source, were analyzed for carbon, hydrogen, nitrogen, sulfur, phosphorus and water content. Samples were sent to the Microanalysis Laboratory - Office of Research Services, University of Massachusetts - Amherst for these analyses.

A modified Pregl-Dumas procedure was used to measure the percent of carbon, hydrogen and nitrogen in the sheath investments. Analyses were performed on a Perkin-Elmer elemental analyzer, model 240 following standard procedures. Sulfur content was determined by decomposing lyophilized sheath material in a Schonizer oxygen flask, followed by the standard barium titration in ethanol using thorin as the indicator reagent. Phosphorus was determined after acid digestion of lyophilized sheath material in concentrated sulfuric and nitric acid by the molybdenum blue procedure. Water content was determined by drying to constant weight at 95 C. All procedures followed standard procedures (Ma & Rittner, 1979).

3.3.2 Water content

The water content of the sheath investment was determined by gravimetric analysis before and after dehydration. Approximately 1 g of sheath gel was added to tared aluminum pans and placed at 95 C for 1 hour. The water loss observed upon heating was an indication of the amount of water embibed by sheath investments.

3.3.3 Sulfate Determination

A microversion of the turbidimetric- barium chloride assay for sulfate in aqueous solutions (Standard Methods, 1980) was developed to determine the presence of sulfate in the lyophilized sheath material. Samples containing .5-1mg of sheath material were hydrolyzed at room temperature in 1ml of 0.05 N and 0.1 N HCl for 20 minutes and at 100 C in 0.5 N HCl for 1 hour. Samples were then centrifuged at 1000 g for 5 minutes and the supernatant isolated and subsequently evaporated over NaOH in vacuo. To each sample was added 1 ml of water and 0.05ml of the conditioning reagent. Approximately 25mg of $BaCl_2$ crystals were added to each vial, vortexed for 1 minute and immediately placed in the spectrophotometer. The maximum absorbance, read at 420 nm, was taken after 4 minutes.

3.3.4 Protein

Several methods were employed in determining the protein content of the lyophilized sheath material. Three colorimetric assays were used: biuret (Gerhardt, 1981), Lowry (Lowry et al., 1951) and a modified ninhydrin method (Rosen, 1957). Spectrophotometry was performed with a Perkin-Elmer double beam spectrophotometer, model PE-200.

The ninhydrin procedure was adopted as the prefered method since it required the least amount of sheath material. A sample containing 70-130 ug was hydrolyzed in 0.5ml of 6 N HCL. Samples were placed in dry 5 ml glass ampules followed by 0.25 ml of water, inorder to hydrate sheath material before adding acid. Ampules were purged with nitrogen for 20 seconds and sealed in an oxygen enriched gas flame prior to hydrolysis at 100 C. After various times of hydrolysis (15 minutes to 24 hours) samples were neutralized with 0.5 ml of 6 N NaOH. The samples were checked with pH paper to assure complete neutralization. To the ampule, .5ml of the acetate-KCN reagent was added and stirred. The mixture was transferred to a 5" test tube, where 0.5ml of a 3 % solution of ninhydrin in ethylene glycol monomethyl

ether was added. Samples were then heated in a boiling water bath for 10 minutes. Immediately after removal from the water bath, 5ml of a 1:1 (v/v) solution of isopropanol and water were added and mixed vigorously. Samples were allowed to cool to room temperature before reading absorbances at 570 nm.

A Beckman amino acid analyzer, model 121 was used to determine the components associated with the protein portion of the sheath investments. Samples containing 5 mg of lyophilized sheath material were hydrolyzed in 6 N HCl for 20 hours in flame sealed, nitrogen pirged, glass ampules. The acid was evaporated over NaOH, in vacuo, with two repetitions of rehydration followed by evaporation. The samples received 1 ml of a 0.2 M sodium citrate buffer to maintain the pH at 2.2. A total volume of 0.5 ml was added to a 56cm x 9mm column containing a type W-1 ion exchange resin. The separation was executed at 55 C, in a 0.2 M sodium citrate buffer, at a flow rate of 70ml/hr, in a 3 buffer system (pH= 3.49, 4.20 & 6.40). Colorimetric detection was performed at 670nm, except for proline which was best detected at 440nm. Ninhydrin was used for color development. All amino acid analyses were performed by the laboratory of Dr. Mokhtar Atallah of

the Department of Food Science Nutrition at the University of Massachusetts - Amherst.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed inorder to isolate the proteinaceous components associated with the sheath investment. Samples containing 5 mg of lyophilized sheath material were hydrated with 1 ml of water. Sample buffer (1 ml), at a pH of 6.8, was added to the sheath material and the vials were placed in a boiling water bath for 3 minutes. The supernatant was collected after centrifugation at 10,000 g for 5 minutes.

A discontinuous gel slab (0.75 mm thick) was prepared between two glass plates (16 x 18 cm). Gels containing 3% (stacking gel, 2cm high) and 10% (running gel, 12cm high) acrylamide, were prepared from a stock solution of 30% acrylamide and 0.8% bisacrylamide. The running gel was prepared with 1.5 M TRIS-HCl buffer, containing 0.4% sodium dodecyl sulfate (SDS) (pH=8.8). The stacking gel contained 0.5 M TRIS-HCl buffer (pH=6.8) and 0.4% SDS. The gels were polymerized chemically, by adding 0.05% TEMED and 0.2% ammonium persulfate. The electrode buffer (pH=8.3) contained 0.3% TRIS-HCl, 1.4% glycine and 0.1% SDS. The sample buffer contained 3% SDS, 0.5% TRIS-HCl buffer (pH=6.8),

10% glycerol and 5% 2-mercaptoethanol. A few crystals of bromophenol brilliant blue dye were added until the desired intensity was achieved. Samples (10-30ul) were carefully layered into the stacking gel wells with pasteur pipets. The remaining volume of the well was filled with sample buffer.

Electrophoresis was carried out with a current of 15 mA/gel until the front reached the bottom of the gel (4-5hrs). The separated proteins were fixed in the gel by emersing the gel slab in 0.1% coomassie blue, containing 45% methanol and 10% glacial acetic acid, overnight. Destaining occurred by repeated washing with a solution containing 10% methanol and 10% glacial acetic acid.

3.3.5 Carbohydrate Analyses

3.3.5a Colorimetric procedures

Preliminary analyses for total carbohydrate, uronic acids, pyruvic acid and O-acetyl groups were determined colorimetrically.

Total carbohydrates were determined by the anthrone assay (Dreywood, 1946). Samples containing 50-100 ug of lyophilized sheath material were hydrated with 1 ml of water. All samples and standards, placed in an ice bath, received 2 ml of a chilled solution of 0.2% anthrone, in 95% sulfuric acid. After vortexing, samples were placed in a boiling water bath for 8 minutes. The reaction was stopped by returning the samples to the ice bath. Samples were allowed to cool to room temperature before reading absorbances at 620 nm.

Uronic acid determinations were performed by the m-phenyl phenol method of Blumenkrantz and Asboe-Hansen (1973). Samples containing 400 ug of lyophilized sheath material were hydrated with 0.4ml of water and placed in an ice bath. To this suspension, 2.4ml of a 0.0125 M solution of sodium tetraborate, in concentrated sulfuric acid was added and heated for 5 minutes in a boiling water bath. The reaction was halted by

transfering samples to an ice bath. Color development was initiated upon addition of 0.04ml of a 0.15% m-phenyl phenol solution in 0.5% NaOH. Absorbances were read after 5 minutes at 520 nm.

Pyruvic acid analysis was performed by the 2,4-dinitrophenylhydrazine method (Lessie & Whiteley, 1969). Samples containing 600-800 ug of lyophilized sheath material were hydrolyzed in 0.5 N sulfuric acid at 100 C for 8 hours. After neutralization of the acid with $BaCO_3$, hydrolysates were evaporated over NaOH in vacuo. To sample residues, 0.5ml of water, followed by 0.5ml of a 0.02% solution of 2,4-dinitrophenylhydrazine, in 0.5N HCl, were added. After 5 minutes, 1ml of a 2N NaOH solution was added for color development. Absorbances were read in 2 minutes at 420 nm.

The method of Hestrin (1949) designed for the determination of acetylcholine, was used to estimate the amount of O-acetyl groups in sheath investments. To a 5ml glass vial containing 1-2 mg of lyophilized sheath material, 0.5ml of water was added to hydrate the sample. To this suspension, 1ml of the hydroxylamine reagent was added. After 1 minute, 0.5ml of a 4 N HCl solution followed by 0.5ml of a 10%

$FeCl_3$ solution, in 0.1N HCl, were added. After centrifugation at 1000 g for 5 minutes, the supernatant of each sample was removed and their absorbances read at 540 nm.

3.3.5b Paper Chromatography

Neutral sugars were tentatively identified via one dimensional paper chromatography. Samples containing 5-10 mg of lyophilized sheath material were hydrolyzed in 1N sulfuric acid at 100 C for various time periods. The hydrolysate was neutralized with barium carbonate and the barium sulfate precipitate was sedimented by centrifugation. The supernatant was transferred to 5ml glass vials and evaporated over NaOH in vacuo. The residue remaining was dissoloved in 100 ul of water and 1-2 ul were applied to the chromatogram using flame constricted capillary tubes (1 drop = 0.12 ul). The solvent system: butanol/pyridine/water (6:4:3 by volume) was run in a descending manner at room temperature (22-24 C) using Whatman # 1 filter paper (24"x 8"). The chromatograms after air drying, were sprayed with alkaline silver nitrate and aniline hydrogen phthalate solutions. Sugar spots were located after heating for 10 minutes at 95 C. Relative R_f

values for sample sugars were compared to those of known standards.

3.3.5c Gas Chromatography

Identification of the sugar components of the sheath investments was made by gas-liquid chromatography. (Swardeker et al., 1965). A dual-column, base line compensation mode was implemented using a Perkin-Elmer, model 900 gas chromatograph, equipped with flame ionization detectors. Separation of sugar derivatives was achieved with stainless steel columns (5.5 ft x 3 mm) packed with 3 % OV-225 on 80 - 100 mesh Supelcoport. The temperature was programmed from 140 to 240 C at 5 C/minute and finally kept isothermal at 240 C until all components had eluted. Nitrogen was used as the carrier gas, which was set at a flow rate of 55ml/minute. Acid hydrolysis of lyophilized sheath investments was performed with 2 N TFA and 1 N H_2SO_4 at 100 C. The samples were centrifuged after various time periods to pellet unhydrolyzed residual material. Samples (2.5mg) hydrolyzed with H_2SO_4 were carefully neutralized with 260mg of $BaCO_3$ (1.3x M $BaCO_3$/M H_2SO_4) inorder to minimize sample loss in excessive $BaSO_4$ precipitate. A small stir bar and 2ml of deionized-distilled water were added to the vials, which were then stirred for 15 minutes. Suspended $BaSO_4$ was centrifuged at 1000g for 5

minutes. The supernatant was transferred to glass vials and evaporated over NaOH in vacuo. A 0.03 % aqueous solution of arabinose (1ml) was added to each sample residue as an internal standard.

A mixture of sugar standards were prepared and together with the sheath samples, reduced to their alditols with sodium borohydride for 2 hours at 22 C (the ratio of sodium borohydride to sheath material was 2:1). Approximately 4 ul of concentrated acetic acid per mg of sodium borohydride was added to decompose excess sodium borohydride. Sodium ions which would interfere with acetylation, were removed by a strong acid cation exchange resin (Dowex 50). Sample and standard solutions (1ml) were cycled through two pasteur pipets (twice/pipet) containing a slurry of the resin beads charged with hydrogen ions. Tygon tubing was attached to the end of the pasteur pipets and pinched with a screw clamp, thereby maintaining a flow rate of approximately 0.3 ml/minute. Resin beads received several water rinses, totalling 2 ml, to promote complete recovery of sample material. Sample and standard solutions, free of sodium ions, were evaporated over NaOH in vacuo. Borate ions were then removed by adding 0.25ml of super dry methanol,

followed by evaporation in a stream of nitrogen. This step was repeated four times to achieve complete removal of borate. Acetylation of the sample and standards was achieved by heating the alditols for 1 hour at 100 C in 0.4ml of pyridine and 0.4ml of acetic anhydride. The solvent was blown off in a stream of nitrogen and depending upon the sample size, 50-100ul of chloroform was then added. Subsequently, 0.5-1 ul of each preparation was injected into the gas chromatograph. Sample peaks were identified by comparing relative peak distances (R_f-arabinose) to those of known sugar standards.

A procedure developed for the simultaneous analysis of aldonic acids and aldoses (Lehrfeld, 1985) was adopted for derivitization of uronic acids. To the evaporated hydrolysates, 0.5-1ml of a 0.1 M sodium carbonate solution was added to protect the carboxyl group on the uronic acid from reduction by sodium borohydride. This solution was allowed to react for 1 hr at 30 C. Procedures for reduction, sodium and borate ion removal were similar to those used for neutral sugars. Following borate ion removal, however, uronic acids were converted into uronolactones by heating at 85 C in vacuo for 2 hours. The uronolactones were then

converted into N-propyluronamides by heating for 30 minutes at 55 C, in 0.4ml of pyridine and 0.4ml of N-propylamine. The sample was evaporated in a stream of nitrogen and acetylation was achieved in a similar fashion to that previously described. With this procedure, uronic acids and aldoses are converted to N-propyluronamide acetates and alditol acetates, respectively.

3.3.5d Mass Spectrometry

Confirmation of neutral sugars, identified by gas chromatography, was accomplished by separating the alditol acetate derivatives with a BD-5 (J & W Scientific) fused silica capillary column (60m x 0.33 mm i.d., coated with a 0.25 um liquid phase) and observing their mass spectra. Alditol acetate derivatization was performed on a sample containing 5 mg of lyophilized sheath material, collected from cultures grown without a combined nitrogen source and on a standard sample containing six known neutral sugars (0.3 mg/ml each of: rhamnose, arabinose, xylose, mannose, galactose and glucose). Both samples received a 1:50 dilution preceding injection (3 ul) into a Hewlett-Packard GC-mass spectrometer, model HP 5985,

coupled by an open split interface. Injection port and manifold temperatures were set at 250 C. Column head pressure and the source temperature were 15 psi and 160 C, respectively. The temperature program chosen was 80 (held for 1 minute) to 110 C at 6 C/minute, followed by 110 to 300 C at 3 C/minute. Electron impact (EI) and chemical ionization (CI) modes were utilized. An ionization potential of 70 eV and a solvent delay (SD) of 20 minutes were used for EI. Isobutane was used for CI, with an ionization chamber pressure of 2×10^{-4} Torr. The instrument was tuned with perfluorotributylamine. All GC-mass spectrometry was performed by Dr. Thomas Potter of the Massachusetts Agricultural Experiment Station, University of Massachusetts - Amherst.

3.4 Sheath Investment Porosity

The porosity of the sheath investment of Gloeothece ATCC 27152 was estimated by determining the degree to which a series of dextran polymers diffused into the sheath investment volume of a pellet of whole cells. Seven standards were obtained from the Sigma Chemical Co., with known average molecular weights, that ranged from 9000-465,000 and Stokes radii of 2.3-14.0 nm. Measurement of the total carbohydrate content of the dextran solutions (by the anthrone test), before and after exposure to the cells, provided an estimate of the limit of penetrability of the sheath investment.

Cells were cultured without a combined nitrogen source, in a 15 liter glass carboy for 28 days. After settling the culture and removing the media, the cells were centrifuged at 250g to remove excess water. Exactly 3ml of a 0.5% dextran solution was added to a 30ml centrifuge tube containing 1.0 gm of ensheathed cells. The tubes were capped, covered with foil and slowly rotated (end over end) at 10 C for 15 hours. The cells were allowed to settle for 3 hours, after which the supernatant was collected by pipet. Supernatant and initial standards were diluted 1:100ml and the total

carbohydrate content was determined on 1ml from each.

3.5 Heavy Metal Binding

The heavy metal binding capacity of sheath investment material, collected from cultures grown with and without sodium nitrate, was determined by exposing a series of sheath samples to varying concentrations of cadmium ions (cadmium nitrate). Exactly 3ml from the metal solutions (25, 50, 75, 100, 150 and 200 ug/ml) were added to 5ml glass vials, containing 2mg of lyophilized sheath material. In order to achieve maximal exposure to the metal ions, each vial was vortexed 5 seconds every 5 minutes, for a total of 30 minutes. The sheath-metal suspension was centrifuged at 1000g for 5 minutes. Supernatant and standard solutions were diluted 1:25ml. Two drops of concentrated nitric acid (instrument pure grade) were added to each volumetric flask before bringing each flask to the appropriate volume with deionized-distilled water. Cadmium concentrations were determined by Irene Ellis in the laboratory of Dr. Thomas Zajicek, Chemistry Department, University of Massachusetts. A Perkin-Elmer ICP model 6500 atomic emmission spectrophotometer was used for the analyses.

CHAPTER FOUR

RESULTS

4.1 Cultivation of Gloeothece ATCC 27152

The growth of axenic cultures of Gloeothece ATCC 27152 was slow and arduous, considering the procaryotic nature of its structure and mode of division. Growth rate studies revealed generation times of 3-5 days (72-120 hrs) depending upon the type of medium employed (Table 4). Growth curves (in triplicate) of Gloeothece ATCC 27152, grown in medium BG-11 with (BG-11-NO_3) and without (BG-11-no NO_3) a combined nitrogen source ($NaNO_3$) and supplemented with 100mg/l of $NaHCO_3$ (BG-11-$NaHCO_3$), are presented in figures 1a & b, 2a, b & c and 3a, b & c, respectively. Culture C of cells grown in medium BG-11-NO_3 failed to grow. An average generation time was consequently based upon the growth of only two cultures.

Maximum growth rates were achieved with medium BG-11-$NaHCO_3$. Cells grown on media BG-11-NO_3 and BG-11-no NO_3 displayed longer generation times and lower growth yields (Table 4).

Microscopic observation of cultures at the close of the growth study revealed a distinct increase in the number of cells per sheathed aggregates in cultures grown with medium BG-11-$NaHCO_3$. Cultures grown on media BG-11 with and without a combined nitrogen source possessed larger sheath to cell volume ratios. On the average these cultures contained 4-8 cells per aggregate, while BG-11-$NaHCO_3$ grown cultures possessed 16-24 cells per aggregate.

Although the growth rate of Gloeothece ATCC 27152 with or without a combined nitrogen source was similar, a disparity occurred in sheath size. The sheath investment of cells grown on medium BG-11-no NO_3 (fixing atmospheric nitrogen) appeared more voluminous and distinct compared to cells grown on medium BG-11-NO_3 (assimilating $NaNO_3$) (Figures C & D).

4.2 Sheath Investment Removal

The rotary ball mill provided the best means for separating the external sheath investment from the cell aggregates. Removal of the sheath investment of Gloeothece ATCC 27152 was best achieved with a rotary ball mill. While this procedure was quite effective at obtaining satisfactory yields of cell-free sheath investments, some damage did occur. A spectrophotometric scan of the milled sheath suspension revealed the presence of chlorophyll a and the phycobilin pigments, phycocyanin and phycoerythrin (Figure 4). High speed centrifugation (38,000g) separated the water soluble phycobilin pigments (Figure 5) molecules. Removal of the latter pigment was achieved after treating the sheath material with lysozyme and SDS. Gravimetric analysis of total lipid content indicated that the crude sheath preparation (prior to lysozyme/SDS treatment) contained only negligible amounts of total lipid soluble material (0.53% and 0.36% for sheath material before and after lysozyme/SDS treatment).

Yields of cell-free sheath investment material were greatest when material for ball milling was obtained

from cultures grown under nitrogen fixing conditions. The larger ratio of sheath investment to cell volume facilitated removal procedures. Therefore, despite the greater growth yields achieved with medium BG-11-$NaHCO_3$ and media BG-11-NO_3, BG-11-no NO_3 provided more amenable culture material for ball milling. Approximately 15 liters of an exponentially growing culture (BG-11-no NO_3) of Gloeothece ATCC 27152, provided 3 gm of dehydrated cells and 150mg of freeze dried sheath investment material (FDS).

4.3 Microanalysis Laboratory Analyses

Several sheath samples (collected from cultures grown with and without a combined nitrogen source) were sent to the Microanalysis Laboratory at the University of Massachusetts to be tested for carbon-hydrogen-nitrogen, phosphorus, sulfur and water content (Table 5). On 7/3/84, two samples of sheath material collected from cultures grown on a combined nitrogen source were analyzed for carbon-hydrogen-nitrogen. The first represented wet sheath gel, while the other was sheath gel dried over H_2SO_4 in a dessicator. The remaining samples were

freeze dried sheath (FDS) material before (-G) and after (-W) lysozyme and SDS treatments. The subscripts, G and W referred to the color (green or white) of the sheath material after lyophilization.

4.4 Water Content

The water content of the wet sheath gel before lysozyme and SDS treatments, as well as that obtained from mutant and wild type cells of Gloeothece ATCC 27152, is presented in Table 6. The sheath gel, isolated from cultures grown without a combined nitrogen source (99.8% water content), was apparently more hydrated than the material collected from cultures grown with $NaNO_3$ (99.5% water content).

4.5 Sulfate Content

The sulfate content of lyophilized sheath investments is presented in Table 7. Sheath investments, collected from cultures grown with and without a combined nitrogen source, contained approximately equal amounts of sulfate. Weak acid hydrolysis (0.5N HCl for 1hr @ 100 C) indicated that

FDS-G-NO_3 material contained 34% less sulfate than FDS-G-no NO_3 material. When stronger hydrolytical conditions were employed, sulfate values matched those theoretically predicted from the total sulfur analysis. To determine if residual SDS reacted with the gravimetric sulfate test, a SDS sample containing 50ug of sulfate, was hydolyzed and tested for liberated sulfate content. While a slight precipitate was formed, it was not comparable to that formed with the sulfate standard and sheath samples. The particle size of the precipitate was larger, causing premature sedimentation. When a similar SDS sample was hydrolyzed for longer periods with 1 N HCl, virtually all of the sulfate was detected.

4.6 Protein Analyses

4.6.1 Colorimetric Procedures

Several colorimetric assays were utilized in estimating the protein content of the sheath investment material. Data from the various procedures is listed in Table 8. The Biuret and Lowry protocols, which utilized a NaOH extraction, detected a protein content of 5-6%. These assays were performed on sheath material not treated with lysozyme and SDS (FDS-G-NO_3).

A modified ninhydrin method was adopted as the preferred method, primarily due to its greater sensitivity. A hydrolysis series showed maximum release of protein (6.3%) had occurred between 4-8 hours with FDS-W-no NO_3 material (Table 9). A comparison of the protein content of the various types of sheath material indicated that FDS-W-NO_3 contained 7.6-7.9%, while FDS-W-no NO_3 possessed a value of 4.0-4.5%. Since hydrolysis lasted for only 3 hours, these results can only represent relative proportions and not absolute protein values. Lysozyme SDS treated sheath material (FDS-W-no NO_3) possessed 48% less protein than similar material not treated with lysozyme and SDS

(FDS-G-no NO_3). Based upon colorimetric analysis, the sheath investment of Gloeothece ATCC 27152 contained a protein content of approximately 6-10%, depending upon whether the cultures fixed atmospheric nitrogen or assimilated combined nitrogen, respectively.

4.6.2 Amino Acid Analyzer

The amino acid composition of the protein portion of the sheath investment collected from cultures grown with a combined nitrogen source is presented in figures 6 through 11. A 42% decrease was observed in total peak area of the individual amino acids when sheath material was treated with lysozyme and SDS (Table 10b).

4.6.3 Electrophoresis

Results from electrophoretic analyses performed on FDS-W-no NO_3 sheath material is presented in figures 12 and 13. Figure 12 represents a trial run which utilized exposable sheath material (ineffectively treated with lysozyme and SDS) to estimate the portion of protein extract to be applied. Extracts (1ml) of sheath material (A = 5.00mg and B = 4.98mg) were

applied to the gel in 10 and 20 microliter volumes. Five major protein bands were observed. The molecular weights of these bands were estimated to be 51,000, 56,000, 32,000, 21,500, and 14,300, based upon a series of standards run through each gel. A second analysis was performed using 4.984mg of FDS-W-no NO_3 material, effectively treated with lysozyme and SDS. Along with this sample, sheath material remaining from the initial trial run was extracted a second time and run with the above material. Both samples were applied in 30ul volumes and run under the same conditions as before (Figure 13). While the sheath sample that was effectively treated with lysozyme and SDS possessed the two sharp 51,000 and 56,000 bands, the 32,000 band was absent and the 21,500 and 14,300 bands were less intense. Interestingly, the re-extracted sheath sample possessed the same bands as before at relatively the same level of intensity.

4.7 Carbohydrate Analyses

4.7.1 Colorimetric Assays

4.7.1a Total carbohydrates

Average total carbohydrate values for FDS-W-NO_3 (55.4% S.D.= 0.78) and FDS-W-no NO_3 (41.0% S.D.=0.46) indicated that cells fixing atmospheric nitrogen produced sheath investments with 26% less total carbohydrate material (Table 11).

4.7.1b Uronic acids

A 21% increase in uronic acid content was detected in FDS-W-no NO_3 compared to FDS-W-NO_3. Values averaged 7.6% (S.D.= 0.18) and 6.0% (S.D.= 0.16), respectively (Table 11).

4.7.1c O-acetyl

The content of O-acetyl groups in the two types of sheath material is presented in Table 12. An equivalent molar relationship between acetyl choline and O-acetyl was assumed. Conversion of micromoles to micrograms facilitated the determination of the percent composition of O-acetyl in the sheath material. The

percent content of O-acetyl detected in FDS-W-NO_3 was 3.07% (S.D.=0.007). This was 29% greater than the 2.19% (S.D.= 0.010) value detected in FDS-W-no NO_3 material.

4.7.1d Pyruvic acid

The pyruvic acid content of FDS-W-no NO_3 is presented in Table 13. The hydrolysis series showed maximum release of pyruvate occurred 8 hours after acid hydrolysis. This corresponded to a pyruvic acid content of 2.28%.

4.7.2 Paper Chromatography

Paper chromatographic analysis of sheath material collected from cultures grown on a combined nitrogen source revealed a total of six spots (Table 14). Spots 4 and 6 stained pink when sprayed with aniline hydrogen phthalate, indicating pentose sugars. The relative distances (R_f) of spots 4 and 5 correlated well with the standards xylose and rhamnose. Spots 1, 2, 3 and 6, however, could not be identified beyond the hexose/pentose designation. While the R_f of the arabinose standard was comparable to that of spot 2,

the first three spots stained grey when sprayed with aniline hydrogen phthalate, thereby indicating a hexose ring structure rather than a pentose. The first three spots migrated with the standards glucose, galactose and mannose but a precise identification could not be determined.

4.7.3 Mass Spectrometry

Electron impact mass spectroscopic analysis resulted in uncharacteristic m/e fragments (mass to charge). Consequently, identification of the neutral sugar derivatives of sheath material collected from cultures fixing atmospheric nitrogen was not possible. The primary fragment, m/e 43, was the acetylium ion:

$$H_3C-C=O$$

This ion is characteristic of spectra of alditol acetate derivatives. An unknown peak at scan number 227 of figure 14A corresponded to the unknown component first observed in the earlier GC chromatograms. Figure 14C contains a m/e fragment representative of an acetoxyl group at C-1 and a methoxyl group at C-2:

$$\begin{array}{l} CH_2\text{-}OAc \\ | \\ CH\text{-}OMe \end{array}$$

and is presumably that of 2-O-methyl xylose.

Chemical ionization mass spectrometry, using isobutane provided information on the molecular weight of the neutral sugar derivatives. The largest spectral peak from each component represents the protonated molecular ion, minus 60 mass units (due to the loss of acetic acid). The molecular ion is 60 mass units larger

than this primary fragment but is usually present in lower abundance. The alditol acetate derivatives of 2-O-methyl xylose, rhamnose, arabinose, xylose, mannose, galactose, and glucose have the following molecular weights: 334, 376, 362, 362, 434, 434, 434 and 434.

The unknown peak occurring at scan number 91 in figure 16A, possessed a different molecular ion abundance. The M-60 fragment, at mass 229 was much less than the molecular ion at mass 289. A second unknown peak, occurring at scan number 236 in figure 16E, possessed a molecular weight similar to a pentose sugar derivative but the minor m/e fragments did not resemble any of the related neutral sugars.

4.7.4 Gas chromatography

The polysaccharide fraction of lyophilized sheath investments, collected from cultures grown with and without a combined nitrogen source, contained a total of 10 sugar residues. These included six neutral sugars (xylose, 2-O-methyl xylose, rhamnose, mannose, glucose and galactose) and three uronic acids (mannuronic, glucuronic and galacturonic acid). There was no difference in the type of sugar molecules comprising FDS-W-NO_3 and FDS-W-no NO_3 material.

The area of each peak was divided by the total peak area for the sample giving a relative estimate for the percent content of each sugar residue. This later value was in turn used to determine the component ratio. The relative proportion of each neutral sugar, depended upon the duration of acid hydrolysis (Tables 15 through 18). The FDS-W-NO_3 material possessed mannuronic, glucuronic and galacturonic acids in the ratio of 1:1:3, respectively (Table 17). When grown under nitrogen fixing conditions, however (Table 18), the proportion of mannuronic acid was much less (ratio = 1:3:4). Despite this variation in proportions, there appeared to be only slight quantitative differences in

the uronic acids detected between the two sheath investment preparations.

A seventh unidentified sugar residue was detected preceeding the rhamnose peak when commercially prepared OV-225 was used (Figure 18a). Despite the dual column compensation mode employed, column bleed caused considerable baseline drift. Packing material prepared with a different source of OV-225 (with 80-100 mesh Suplecoport), yielded a more stable baseline. However, the unidentified component was no longer detectable with the manually prepared column packing (Figure 18b).

The effects of acid hydrolysis with 2N TFA at 100 C for 35 and 45 minutes, 1 and 6 hours, on the FDS-W-no NO_3 material is depicted in figures 19 through 22. Apparently, galactose and glucose required longer heating times before being completely liberated (Table 16). The proportions of rhamnose, 2-O-methyl xylose, xylose and mannose after each hydrolysis time were relatively the same.

Hydrolysis of the sheath material at 100 C in 1 N H_2SO_4 for various time periods, (Figures 23 through 30) indicated that maximum release of uronic acid residues occurred after 10 hours (Tables 17 & 18). Further heating did not increase their yield.

Degradation of neutral sugars resulted from 20 hour hydrolysis (Figure 30).

Originally the sheath investment material was hydrolyzed with 2 N TFA (trifluoroacetic acid) at 100 C. The volatility of this acid facilitated its rapid removal without risking substantial losses during salt neutralization. While acetylation of neutral sugars proceeded satisfactorily, substantial interference occurred during uronic acid derivatization. The process of lactone conversion (Heating @ 85 C for 2 hrs in vacuo), in conjunction with N-propyl amine addition, interacted unfavorably with residual TFA remaining in the sheath hydrolyzate. Figure 31 shows the proliferation of aberrant peaks characteristic of excessive acid degradation (see the 20 hour 1N H_2SO_4 hydrolysis, Figure 30). When standards were hydrolyzed with 2 N TFA, derivatization was not affected. Acetylation of sheath material immediately following lactone formation was likewise not affected. Difficulty in derivatization only occurred when lactone formation preceeded N-propyl amine addition. Attempts at removing the residual TFA were not successful (one evaporation over NaOH in vacuo followed by 3 lyophilizations each followed by hydration with 1ml of

water). Consequently, acid hydrolysis with 2 N TFA was replaced with 1 N H_2SO_4. Prudent neutralization with $BaCO_3$ permitted excellent derivatization with virtually no loss in sample hydrolyzate. Comparison of peak areas from sheath material hydrolyzed for 1 hour in 2 N TFA and 1 N H_2SO_4 suggested that 2 N TFA was the stronger hydrolysis reagent (Table 15 and Figures 32 and 18b).

Figure 33 represents the alditol acetate derivatives of the sugar standards: rhamnose , 2-O-methyl xylose, arabinose, xylose, mannose, galactose, glucose, mannuronic lactone, glucuronic acid and galacturonic acid.

4.8 Sheath Investment Porosity

The optical density of the dextran solutions before and after exposure to the cell pellets is recorded in Table 19. Percent uptake values (S^W) based upon the weight of the cells were calculated from the equation presented by Scherrer and Gerhardt (1971):

$$S^W = (\text{Solution vol./cell wt.})(\text{initial OD/final OD})x100$$

Due to the presence of interstial water in the cell pellet, a corrected weight-percent uptake value (R^W) was calculated:

$$R^W = (S^W\text{-sol} - S^W\text{-in})/(100 - S^W\text{-in})$$

S^W-sol = percent uptake for each solute

S^W-in = percent uptake of a high molecular weight standard that represents only the interstitium

The uptake or more accurately the diffusion pattern of the dextran solutions is presented in Figure 34. Two inflection points, one at a molecular radius of 4nm and the other at 8nm, represent the limit of penetrability of the cell wall and sheath investment, respectively.

4.9 Heavy Metal Binding Capacity

Cadmium concentrations of supernatant and initial solutions are presented in Table 20. Sheath investments collected from cultures assimilating sodium nitrate bound 27% more cadmium (per mg of sheath investment material) than sheath investments collected from cultures fixing atmospheric nitrogen (Figure 35).

Table 1

Summary of the chemical composition of cyanobacterial glycocalyx and slime layers.

Cyanobacterium	External Layer Components										References
	neutral sugars							uronic acids			
	glu	gal	man	rha	ara	xyl	rib	glu	gal	man	
Nostoc muscorum	+	+	-	+	+	+	+	(2 unknowns)			Biswas (1957)
Nostoc linkia	+	+	-	+	+	+	-	(unidentified)			Kokyrsta & Chekoi (1972)
Nostoc sp.	+	-	-	-	-	+	-	+	-	-	Mehta & Vaidya (1978)
Nostoc sp.	(35%)		-	10%	-	25%	-	(30%)			Hough et al (1952)
Anabaena cylindrica	5	-	-	-	-	+	-	+	-	-	Bishop et al (1954)
Anabaena cylindrica	47	6	25	-	-	21	-	-	-	-	Dunn & Wolk (1970)
Anabaena flos-aquae	88	-	-	-	-	39	3	1	-	-	Moore & Tischer (1965)
Anabaena flos-aquae											
neutral	8	-	-	-	-	1	-	-	-	-	Wang & Tischer (1973)
acidic	6	-	-	-	-	1	1	(10%)			
Anacystis nidulans	60	14	20	-	-	-	-	-	-	-	Sangar & Dugan (1972)

Table 1

Table 2

Generic classification scheme for cyanobacteria adopted from Rippka et al.,(1979)

<table>
<tr><td>Unicellular:
cells single or forming colonial aggregates held together by additional outer cell wall layers.</td><td colspan="3">Reproduction by binary fission or by budding. I

Reproduction by multiple fission giving rise to small daughter cells (baeocytes) or by both multiple and binary fission. II</td></tr>
<tr><td rowspan="3">Filamentous:
a trichome (chain of cells) which grows by intercalary cell division.</td><td rowspan="3">Reproduction by random trichome breakage by formation of hormogonia and (section IV & V only) sometimes by generation of akinetes.</td><td>Trichome always composed of vegetative cells.</td><td>Division in only one plane. III</td></tr>
<tr><td rowspan="2">In the absence of combined nitrogen trichome contains heterocysts some also produce akinetes.</td><td>Division in only one plane. IV</td></tr>
<tr><td>Division in more than one plane. V</td></tr>
</table>

Table 2

Table 3

Macro and micro nutrient concentrations used in preparing medium BG-11.

Macronutrients	mg/L
$NaNO_3$	1500.00
K_2HPO_4	40.00
$MgSO_4 - 7H_2O$	75.00
Na_2CO_3	20.00
$CaCl_2$	27.00
Na_2EDTA	1.00
citric acid	6.00
ferric ammonium citrate	6.00

Micronutrients	mg/L
H_3BO_4	2.86
$MnCl_2 - 4H_2O$	1.81
$ZnSO_4 - 7H_2O$	0.22
$NH_4MoO_4 - 4H_2O$	0.29
$CuSO_4 - 5H_2O$	0.80
$Co(NO_3)_2 - 6H_2O$	0.05

Table 3

Table 4

Optical density of cultures grown on medium BG-11 with and without a combined nitrogen source and supplemented with 100mg/L of $NaHCO_3$.

Day	Treatment							
	$BG\text{-}11_{NaHCO_3}$			$BG\text{-}11_{no\ NO_3}$			BG-11	
	A	B	C	A	B	C	A	B
0	.023	.055	.035	.043	.066	.047	.055	.055
1	.013	.040	.026	.023	.044	.012	.044	.050
2	.014	.049	.030	.017	.046	.010	.057	.067
3	.030	.070	.040	.028	.054	.022	.070	.071
4	.027	.075	.050	.037	.056	.031	.078	.075
5	.035	.085	.070	.039	.078	.045	.085	.082
6	.055	.095	.080	.068	.090	.061	.105	.090
7	.073	.100	.102	.093	.096	.080	.130	.100
8	.100	.130	.110	.105	.111	.100	.130	.115
9	.120	.155	.135	.132	.144	.129	.160	.150
10	.190	.190	.170	.152	.162	.146	.180	.173
11	.250	.220	.210	.170	.182	.160	.200	.205
12	.326	.260	.275	.197	.192	.187	.240	.240
13	.380	.320	.323	.207	.234	.205	.260	.265
14	.410	.360	.385	.219	.220	.243	.300	.325
15	.470	.400	.440	.213	.220	.248	.320	.370
16	.520	.450	.500	.220	.225	.273	.330	.430
Generation time(days)	2.8	3.6	3.3	5.1	5.0	3.6	5.0	4.2
average		3.2			4.6		4.6	

Table 4

Table 5

Microanalysis Laboratory analyses on FDS-NO_3 and FDS-no NO_3 material for carbon-hydrogen-nitrogen, phosphorus, sulfur and water content.

Analysis	Date					
	7/3/84		11/14/84	7/18/85		3/20/86
	sheath gel	sheath gel dried over H_2SO_4	FDS-G_{NO_3}	FDS-G_{NO_3}	FDS-W_{NO_3}	FDS-$W_{no\ NO_3}$
carbon	0.58	28.05	29.42	29.01	29.91	30.99
hydrogen	11.09	6.30	5.11	4.69	4.75	4.60
nitrogen	0.49	1.79	2.19	1.81	1.60	1.48
phosphorus	-	-	0.12	-	-	0.06
sulfur	-	-	5.78	5.15	6.23	5.30
water	-	-	4.30	-	-	4.30

Table 5

Table 6

Water content of sheath gel before lysozyme/SDS treatment, performed under several drying conditions.

DATE	MATERIAL	WET WT. (mg)	DRY WT. (mg)	% WATER	DRYING TIME (hrs)	TEMP. (C)
3/14/84	FDS-G$_{NO_3}$	71.8	0.3	99.6	18	102
3/16/84	FDS-G$_{NO_3}$	309.5	1.5	99.5	18	102
5/3/84	FDS-G$_{NO_3}$	224.8	1.1	99.5	12	90
7/6/84	FDS-G$_{NO_3}$	420.8	7.7	98.2	48	Dessication over H_2SO_4
7/27/84	mutant cells	112.1	23.7	78.9	72	Dessication
7/27/84	wild type cells	376.8	15.5	95.9	72	Dessication
7/27/84	FDS-G$_{NO_3}$	541.3	6.2	98.9	72	Dessication
5/28/85	thawed FDS-SDS	297.0	2.3	99.2	5/16	95/60
5/28/85	thawed FDS-SDS	269.2	1.8	99.3	5/16	95/60
10/21/85	FDS-G$_{no\ NO_3}$	761.4	1.5	99.8	43	95
10/21/85	FDS-G$_{no\ NO_3}$	855.2	1.7	99.8	43	95
10/21/85	FDS-G$_{no\ NO_3}$	864.1	1.9	99.8	43	95
10/21/85	FDS-G$_{no\ NO_3}$	843.0	1.7	99.8	43	95

Table 6

Table 7

The optical density and percent sulfate content of lyophilized sheath material hydrolyzed for 1 hour at 100 C in 0.5 N HCl. (*) refers to material extracted in 2:1 chloroform/methanol.

Sheath Investment material	weight(ug)	Optical Density (420nm)	Sulfate weight(ug)	%	average %	S.D.
FDS-$G^{*}_{NO_3}$	460	.36	39	8.5	8.7	.57
	460	.40	43	9.3		
	535	.42	46	8.6		
	420	.30	33	7.9		
	480	.40	44	9.2		
FDS-$G^{*}_{NO_3}$	462	.38	42	9.1		
FDS-W_{NO_3}	534	.60	66	12.4	12.8	.68
	578	.64	70	12.1		
	552	.69	75	13.6		
	512	.61	67	13.1		
FDS-G_{NO_3}	452	.72	79	17.5 (28 hour hydrolysis)		
FDS-$G^{*}_{no\ NO_3}$	600	.72	79	13.2	13.2	.40
	546	.64	70	12.8		
	546	.67	74	13.6		
FDS-$G_{no\ NO_3}$	544	.63	69	12.7		
FDS-$W^{*}_{no\ NO_3}$	572	.70	77	13.5		
FDS-$W_{no\ NO_3}$	577	.75	83	14.4		
FDS-$G_{no\ NO_3}$	506	.71	78	15.4 (28 hour hydrolysis)		

Table 7

Table 8

Colorimetric analysis of FDS-NO_3 and FDS-no NO_3 material before (G) and after ($\bar{W}$) lysozyme/SDS treatments, using Biuret, Lowry and ninhydrin assays.

DATE	METHOD	MATERIAL	PROTEIN (ug)	SAMPLE (ug)	% PROTEIN
1/18/85	Biuret	FDS-G_{NO_3}	1150.0	22,000.0	5.2
1/22/85	Lowry	FDS-G_{NO_3}	600.0	10,000.0	6.0
12/11/85	Ninhydrin	FDS-$W_{no\ NO_3}$	4.5	99.0	4.5
12/11/85	Ninhydrin	FDS-$W_{no\ NO_3}$	4.8	119.0	4.0
12/11/85	Ninhydrin	FDS-W_{NO_3}	8.5	112.0	7.6
12/11/85	Ninhydrin	FDS-W_{NO_3}	9.0	128.0	7.0
12/11/85	Ninhydrin	FDS-$G_{no\ NO_3}$	9.5	114.0	8.3
12/11/85	Ninhydrin	FDS-$G_{no\ NO_3}$	8.5	105.0	8.1

Table 8

Table 9

Hydrolysis series of FDS-W-no NO_3 material in 6 N HCl at 100 C from 15 minutes to 24 hours for protein content by the ninhydrin assay.

TIME (hr)	PROTEIN (ug)	SAMPLE (ug)	% PROTEIN
.25	2.0	132	1.5
.50	3.0	119	2.5
.75	4.0	104	3.8
1	3.5	90	3.9
2	3.0	72	4.2
3	4.5	99	4.5
4	4.5	71	6.3
8	5.0	79	6.3
12	5.5	94	5.9
14	7.3	128	5.7
18	7.5	133	5.6
20	5.8	103	5.6
22	5.0	120	4.2
24	2.5	99	2.5

Table 9

TABLE 10a

Amino acid composition of FDS-NO_3 material before and after SDS treatment. (% ala refers to the relative percentage of each amino acid compared to alanine)

Peak #	Amino acid	Sheath Material: FDS-W_{NO_3}-SDS (20mg) 2/26/85			FDS-G_{NO_3} (10mg) 2/7/85			Standards 2/7/85	
		cm^2	nM	%ala	cm^2	nM	%ala	cm^2	nM
1	Asp	4.56	188	139	5.52	227	162	2.43	100
2	Thr	2.93	105	78	2.97	107	76	2.78	100
3	Ser	3.44	132	98	2.97	114	81	2.61	100
4	Glu	4.62	122	90	3.96	105	75	3.77	100
5	Pro	0.42	86	64	0.54	110	79	0.49	100
6	Cys	0.00	0	0	0.00	0	0	1.71	50
7	Gly	5.97	188	139	6.60	208	149	3.18	100
8	Ala	4.58	135	100	4.75	140	100	3.39	100
9	Val	3.87	139	103	3.71	133	95	2.78	100
10	Meth	2.88	142	105	1.88	93	66	2.03	100
11	Isoleu	3.20	136	100	3.38	143	102	2.36	100
12	Leu	4.37	190	141	4.15	180	129	2.30	100
13	Norleu	0.00	0	0	0.00	0	0	3.30	100
14	Tyr	1.95	74	55	2.55	97	69	2.64	100
15	Pheala	3.30	136	100	2.74	113	81	2.42	100
16	Hist	0.92	40	19	0.81	35	25	2.30	100
17	Lys	2.66	111	82	2.98	124	89	2.40	100
18	NH_4	7.92	-	-	6.30	-	-	2.20	-
18a	Tryp	0.00	0	0	0.00	0	0	2.07	100
19	Arg	3.08	103	76	3.51	118	84	2.98	100
		60.67	2027		59.32	2047			

Table 10a

Table 10b

Amino acid composition of FDS-NO_3 material before and after lysozyme/SDS treatments.

Peak #	Amino acid	Sheath Material FDS-W_{NO_3} (5mg) 7/19/85			FDS-G_{NO_3} (5mg) 7/19/85			Standards 7/19/85	
		cm^2	nM	%ala	cm^2	nM	%ala	cm^2	nM
1	Asp	2.74	112	110	3.92	160	90	2.45	100
2	Thr	1.26	57	56	2.52	114	64	2.22	100
3	Ser	1.62	85	83	2.60	137	77	1.90	100
4	Glu	2.07	81	79	3.98	155	87	2.56	100
5	Pro	0.05	8	8	0.42	79	44	0.53	100
6	Cys	0.21	8	8	0.02	1	1	1.43	50
7	Gly	2.59	104	102	3.70	149	84	2.48	100
8	Ala	2.28	102	100	3.99	178	100	2.24	100
9	Val	1.51	63	62	2.76	115	65	2.40	100
10	Meth	0.26	9	9	1.91	65	37	2.96	100
11	Isoleu	1.36	51	50	2.93	110	62	2.67	100
12	Leu	2.07	80	78	3.45	134	75	2.58	100
13	Norleu	0.00	0	0	0.00	0	0	2.38	100
14	Tyr	0.81	32	31	1.47	58	33	2.52	100
15	Pheala	1.65	60	59	2.94	108	61	2.73	100
16	Hist	0.42	14	14	0.60	20	11	2.96	100
17	Lys	1.54	51	50	2.64	87	49	3.04	100
18	NH_4	3.50	-	-	5.59	-	-	2.07	-
18a	Tryp	0.00	0	0	0.00	0	0	-	-
19	Arg	2.05	71	70	3.07	107	60	2.88	100
		27.99	988		48.51	1777			

Table 10b

Table 11

Percent total uronic acid and carbohydrate content of FDS-W-NO_3 and FDS-W-no NO_3 material.

Analysis	Date	Material type	(ug)	Component (ug)	(%)	av. %	S.D.
Uronic Acids	7/30/85	FDS-W_{NO_3}	377	22.0	5.8	6.0	.16
			299	18.5	6.2		
			327	14.5	6.0		
			348	21.0	6.0		
	11/25/85	FDS-$W_{no\ NO_3}$	365	28.0	7.7	7.6	.18
			354	27.5	7.8		
			333	24.5	7.4		
			334	25.0	7.5		
Total Carbohydrate	7/29/85	FDS-W_{NO_3}	68	38.0	55.9	55.4	.78
			93	51.0	54.8		
	11/27/85	FDS-$W_{no\ NO_3}$	46	19.0	41.3	41.0	.46
			74	30.0	40.5		
			75	31.0	41.3		

Table 11

Table 12

Percent content of O-acetyl groups in FDS-W-NO_3 and FDS-W-no_3 material.-no NO_3 material.

Material type	(ug)	Acetyl choline (ug)	(uM)	(uM)	O-Acetyl (ug)	%
FDS-$W_{no\ NO_3}$	1680	114	627.4	627.4	37.0	2.20
	1630	110	605.4	605.4	35.7	2.19
	1626	109	599.9	599.9	35.4	2.18
FDS-$G_{no\ NO_3}$	2900	186	1023.7	1023.7	60.4	2.08
FDS-W_{NO_3}	1565	148	814.5	814.5	48.1	3.07
	1698	160	880.6	880.6	52.0	3.06

Table 12

Table 13

Pyruvic acid content of FDS-W-no NO_3 material hydrolyzed in 0.5 N H_2SO_4 at 100 C from 0 to 13 hours.

Time (hr)	Material type	(ug)	Pyruvate (ug)	(%)
00.0	FDS-$W_{no\ NO_3}$	666	00.0	0.00
00.5		683	02.0	0.29
01.0		646	01.6	0.27
01.5		674	02.4	0.37
02.5		645	02.8	0.43
03.0		815	03.2	0.39
06.0		775	05.6	0.72
07.0		692	08.4	1.21
08.0		703	16.0	2.28
13.0		739	13.2	1.79

Table 13

Table 14

Summary of paper chromatographic analyses performed on FDS-W-NO_3 material. Migration distances are recorded in centimeters. Relative R_f values were based upon sample spot #1.

Spot	Distance from origin (cm)				Relative R_f (spot 1)			
	1/29/85	2/1/85	2/7/85	2/7/85	1/29/85	2/1/85	2/7/85	2/7/85
1	14.0	17.5	13.5	13.0	1.00	1.00	1.00	1.00
2	16.0	20.0	15.5	15.0	1.14	1.14	1.15	1.15
3	18.5	22.5	18.0	16.5	1.32	1.29	1.33	1.27
4	20.0	25.5	20.0	19.0	1.43	1.46	1.48	1.46
5	25.0	30.5	25.0	23.0	1.79	1.74	1.85	1.77
6	27.5	34.5	27.5	25.5	1.96	1.97	2.04	1.96
galactose	14.0	17.5	14.0	13.0	1.00	1.00	1.04	1.00
glucose	14.0	-	14.0	-	1.00	-	1.04	-
arabinose	16.0	-	15.5	-	1.14	-	1.15	-
mannose	17.0	-	17.0	16.5	1.21	-	1.26	1.27
fructose	18.0	22.5	17.5	-	1.29	1.29	1.30	-
xylose	20.0	25.5	19.5	19.5	1.43	1.46	1.34	1.50
rhamnose	-	31.0	-	24.5	-	1.77	-	1.89
time (hrs)	16.5	19.0	16.5	15.0				

Table 14

Table 15

Summary of the peak area, percent content and relative ratio of the sugar components, detected by gas chromatography, from FDS-W-NO_3 and FDS-W-no NO_3 material hydrolyzed at 100 C for 1 hour in 2 N TFA and 1 N H_2SO_4.

Component	3/3/86			3/3/86			3/12/86			3/12/86			6/19/86		
	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio
unknown	0.36	9.2	1.0	0.23	9.2	1.0	-	-	-	-	-	-	-	-	-
rhamnose	0.71	18.2	2.0	0.75	18.8	3.0	0.69	23.5	3.0	0.66	22.4	2.5	0.41	21.0	3.0
2-O-methyl xylose	0.35	9.0	1.0	0.29	7.3	1.0	0.21	7.2	1.0	0.25	8.5	1.0	0.15	7.7	1.0
ARABINOSE*	1.27	-	-	1.65	-	-	0.94	-	-	1.16	-	-	2.47		
xylose	0.36	9.2	1.0	0.31	7.8	1.0	0.28	9.6	1.5	0.25	8.5	1.0	0.30	15.4	2.0
mannose	1.05	26.9	3.0	1.07	26.8	4.5	0.80	27.3	4.0	0.75	25.4	3.0	0.63	32.4	4.5
galactose	0.63	16.0	2.0	0.92	23.0	4.0	0.61	20.8	3.0	0.70	23.7	3.0	0.32	16.4	2.0
glucose	0.45	11.5	1.0	0.43	10.8	2.0	0.34	11.6	1.5	0.34	11.5	1.5	0.14	7.2	1.0

sheath material	FDS-$W_{no\ NO_3}$	FDS-W_{NO_3}	FDS-$W_{no\ NO_3}$	FDS-W_{NO_3}	FDS-$W_{no\ NO_3}$ (H_2SO_4)
total cm^2	3.91	4.00	2.93	2.95	1.95
volume injected	.6ul	.6ul	.5ul	.5ul	1ul
hydrolysis time	1 hr.	1 hr.	1 hr.	1 hr.	1 hr.
sample weight	5.033mg	5.026mg	5.032mg	5.026mg	2.60mg
sample weight accounted for	0.92mg	0.73mg	0.94mg	0.76mg	0.12mg
% detected	18.4	14.5	18.6	15.2	4.5

Table 15

Table 16

Summary of the peak area, percent content and relative ratio of the sugar components, detected by gas chromatography, from FDS-W-no NO_3 material hydrolyzed at 100 C for 35 and 45 minutes, 1 and 6 hours in 2 N TFA.

Component	3/12/86			3/12/86			3/12/86			6/4/86		
	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio
rhamnose	0.17	27.0	4.0	1.26	19.8	2.5	0.96	18.7	2.0	0.88	14.5	2.0
2-O-methyl xylose	0.06	9.5	4.0	0.65	10.2	1.5	0.57	11.1	1.5	0.40	6.6	1.0
ARABINOSE*	1.32			2.34			1.05			1.62		
xylose	0.07	11.1	2.0	0.49	7.7	1.0	0.43	8.4	1.0	0.39	6.4	1.0
mannose	0.21	33.3	5.0	2.00	31.5	4.0	1.25	24.4	3.0	0.99	16.3	2.5
galactose	0.08	12.7	2.0	1.20	18.9	2.5	1.20	23.4	3.0	1.82	30.0	4.5
glucose	0.04	6.3	1.0	0.75	11.8	1.5	0.72	14.0	1.5	1.58	26.1	4.0
total cm^2	0.63			6.35			5.13			6.06		
volume injected	.5ul			.5ul			.5ul			.5ul		
hydrolysis time	35 min.			45 min.			1 hr.			6 hrs.		
sample weight	5.05mg			4.98mg			4.99mg			5.07mg		
sample weight accounted for	0.14mg			0.81mg			1.47mg			1.12mg		
% detected	2.8			16.3			29.5			22.1		

Table 16

TABLE 17

Summary of the peak area, percent content and relative ratio of the sugar components, detected by gas chromatography, from FDS-W-NO_3 material hydrolyzed at 100 C for 5, 6, 10 and 15 hours in 1 N H_2SO_4. (* indicates the relative ratio of percent uronic acid values, excluding neutral sugar values)

Table 17

Component	7/3/86			6/26/86			7/3/86			6/26/86		
	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio
rhamnose	1.27	13.7	3.0	1.29	15.2	3.0	0.96	10.8	3.0	1.08	12.8	3.0
2-O-methyl xylose	0.72	7.8	1.5	0.68	8.0	2.0	0.51	5.8	2.0	0.59	7.0	2.0
ARABINOSE*	1.42			1.46			1.01			1.13		
xylose	0.47	5.1	1.0	0.41	4.8	1.0	0.28	3.2	1.0	0.32	3.8	1.0
mannose	1.82	19.7	4.0	1.61	18.9	4.0	1.83	20.7	6.5	1.34	15.8	4.0
galactose	2.81	30.3	6.0	2.46	28.9	6.0	2.78	31.4	10.0	2.80	33.1	9.0
glucose	2.17	23.4	4.5	1.97	23.2	5.0	2.31	26.1	8.0	2.24	26.4	7.0
mannuronic acid	-	-	-	0.03	.3	1.5**	0.04	.5	1.0**	0.03	.4	1.0**
glucuronic acid	-	-	-	0.02	.2	1.0**	0.03	.3	1.0**	0.03	.4	1.0**
galacturonic acid	-	-	-	0.04	.5	2.5**	0.11	1.2	3.5**	0.05	.6	2.0**
total cm^2	9.26			8.51			8.85			8.48		
volume injected	.6ul			.5ul			.6ul			.5ul		
hydrolysis time	5 hrs.			6 hrs.			10 hrs.			15 hrs.		
sample weight	2.69mg			2.77mg			2.58mg			2.62mg		
sample weight accounted for	0.82mg			0.87mg			1.10mg			1.13mg		
% detected	30.3			31.6			42.4			43.0		

Table 18

Summary of the peak area, percent content and relative ratio of the sugar components, detected by gas chromatography, from FDS-W-no NO_3 material hydrolyzed at 100 C for 5, 10, 15 and 20 hours in 1 N H_2SO_4.

Table 18

Component	7/3/86			7/3/86			6/19/86			6/19/86		
	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio	cm^2	%	ratio
rhamnose	1.03	11.6	3.0	1.11	11.9	3.5	2.14	15.5	3.0	0.97	12.0	2.0
2-O-methyl xylose	0.46	5.2	1.0	0.46	4.9	1.5	1.01	7.3	1.0	0.42	5.2	1.0
ARABINOSE*	0.99			0.96			2.15			2.53		
xylose	0.35	3.9	1.0	0.32	3.4	1.0	0.75	5.4	1.0	1.40	17.3	3.0
mannose	1.82	20.5	5.0	1.91	20.6	6.0	2.40	17.3	3.0	1.55	19.1	4.0
galactose	2.99	33.7	8.5	2.75	29.6	9.0	4.31	31.1	6.0	1.76	21.7	4.0
glucose	2.16	24.3	6.0	2.55	27.4	8.0	3.00	21.7	4.0	1.55	19.1	4.0
mannuronic acid	-	-	-	0.02	.2	1.0**	0.03	.2	1.0**	0.07	.9	1.0**
glucuronic acid	-	-	-	0.03	.3	1.5**	0.08	.6	3.0**	0.15	1.8	2.0**
galacturonic acid	0.10	1.1	-	0.16	1.7	8.0**	0.12	.9	4.0**	0.24	3.0	3.5**
total cm^2	8.91			9.31			13.84			8.11		
volume injected	.6ul			.6ul			.5ul			1ul		
hydrolysis time	5 hrs.			10 hrs.			15 hrs.			20 hrs.		
sample weight	2.57mg			2.61mg			2.58mg			2.62mg		
sample weight accounted for	1.35mg			1.45mg			0.97mg			0.48mg		
% detected	52.5			55.6			37.4			18.4		

Table 19

The optical density of dextran polymer solutions used to estimate the limit of penetrability of the sheath investment of Gloeothece ATCC 27152, including percent uptake (S^W) and corrected percent uptake (R^W) values.

Dextrans molecular weight	molecular radius(nm)	Cell Weight (gm)	Optical Density initial	final	S^w	R^w
9000	2.3	1.01	.585	.458		
			.590	.466		
			.585	.457		
			~~.638~~	.462		
			.585	.462		
			.586	.461	80.5	.71
14,700	3.1	1.03	.601	.468		
			.600	.467		
			.600	.470		
			-	.465		
			.603	.462		
			.601	.466	84.4	.77
42,500	4.2	1.01	.648	.527		
			.642	.531		
			.638	.528		
			.651	.530		
			.644	.530		
			.645	.529	65.1	.48
79,500	6.2	1.01	.663	.547		
			.662	.547		
			.658	.551		
			.660	.550		
			.653	.543		
			.659	.548	60.2	.40
153,000	8.4	1.02	.685	~~.592~~		
			.688	.582		
			.685	.582		
			.687	~~.591~~		
			~~.659~~	.582		
			.686	.582	52.6	.29
229,000	9.8	1.00	~~.731~~	.640		
			.714	.641		
			.713	.643		
			.713	.645		
			~~.729~~	.640		
			.713	.642	33.2	00
465,000	14.0	1.01	.678	.595		
			-	.595		
			.679	.595		
			.680	.594		
			.678	~~.602~~		
			.679	.595	41.9	00

Table 19

Table 20

Cadmium ion concentrations before and after exposure to lyophilized sheath investment material.

Sheath Investment (mg)	Cadmium Concentration (ug/ml)	Cadmium Available in 3 ml	Cadmium Remaining in Supernatant	Cadmium Uptake (ug/mg)
$FDS\text{-}G_{no\ NO_3}$				
2.02	11	33	.5	16
2.06	17	51	6	22
2.06	27	81	18	31
2.07	36	108	30	38
2.06	79	237	108	63
2.01	102	306	172	67
2.04	162	486	315	84
2.01	206	618	456	81
$FDS\text{-}G_{NO_3}$				
2.00	25	75	13	31
2.00	50	150	52	49
2.02	81	243	108	67
2.04	115	345	164	89
2.05	163	489	304	90
2.06	217	651	422	111

Table 20

Table 21

A tally of the components of the sheath investment preparations.

Parameter	PERCENT TOTALS FDS-no NO_3	FDS-NO_3
total carbohydrate	41.0	55.4
sulfate	15.4	17.5
protein	6.3	10.9
total uronic acids	7.6	7.6
water	4.3	4.3
O-acetyl	2.2	3.1
pyruvate	2.3	-
	79.1	98.8

Table 21

Figure A

Photograph of Gloeothece ATCC 27152 depicting an average aggregate of 16 cells.

Figure A

Figure B

Photograph of cell free sheath investment material after ball milling.

Figure B

Figure C

Photograph of Gloeothece ATCC 27152 cultured with a combined nitrogen source.

Figure C

Figure D

Photograph of Gloeothece ATCC 27152 cultured without a combined nitrogen source (i.e. fixing atmospheric nitrogen).

Figure D

Figure 1a

Growth curve A of Gloeothece ATCC 27152 cultivated on medium BG-11.

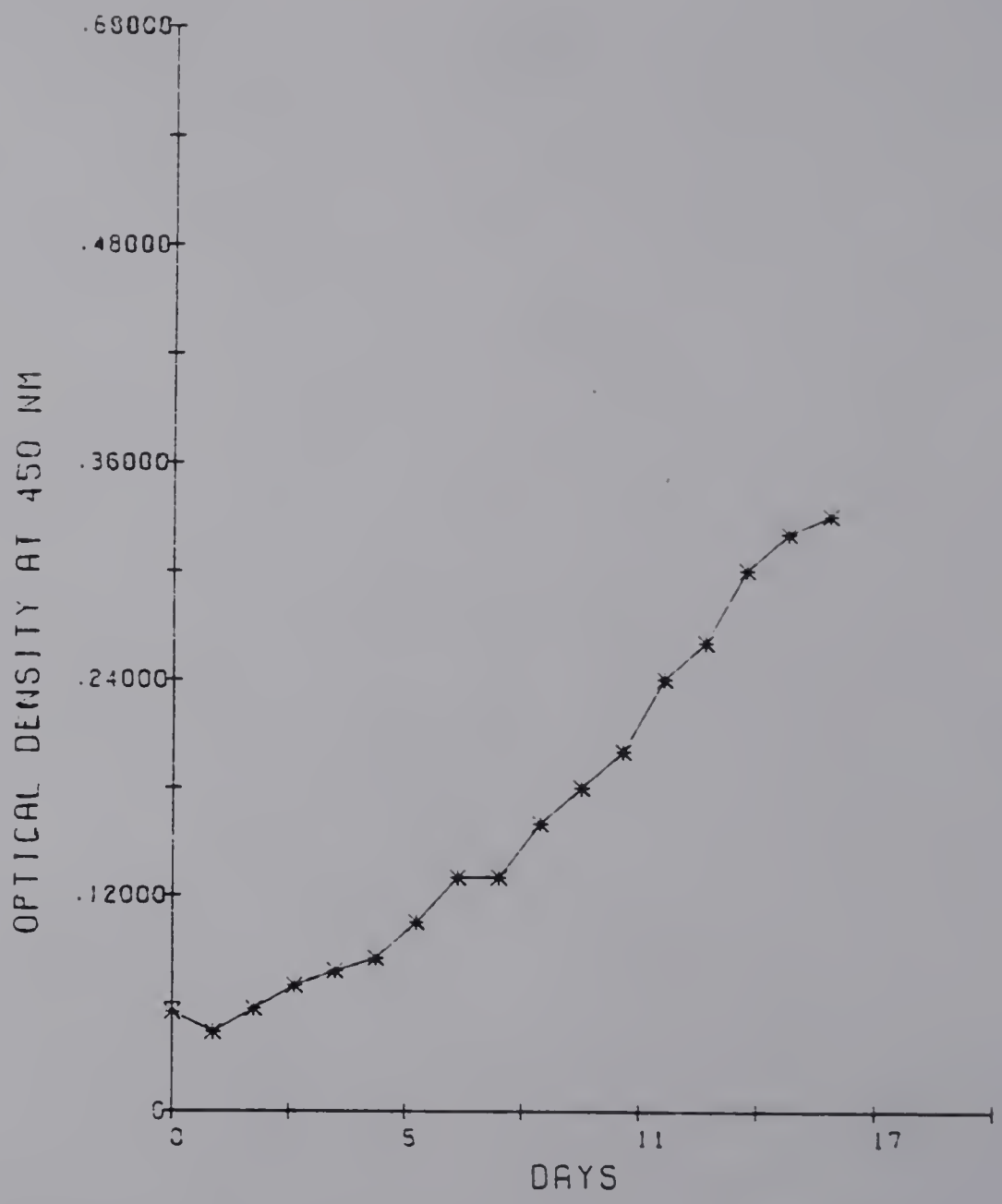

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 1a

Figure 1b

Growth curve B of Gloeothece ATCC 27152 cultivated on medium BG-11.

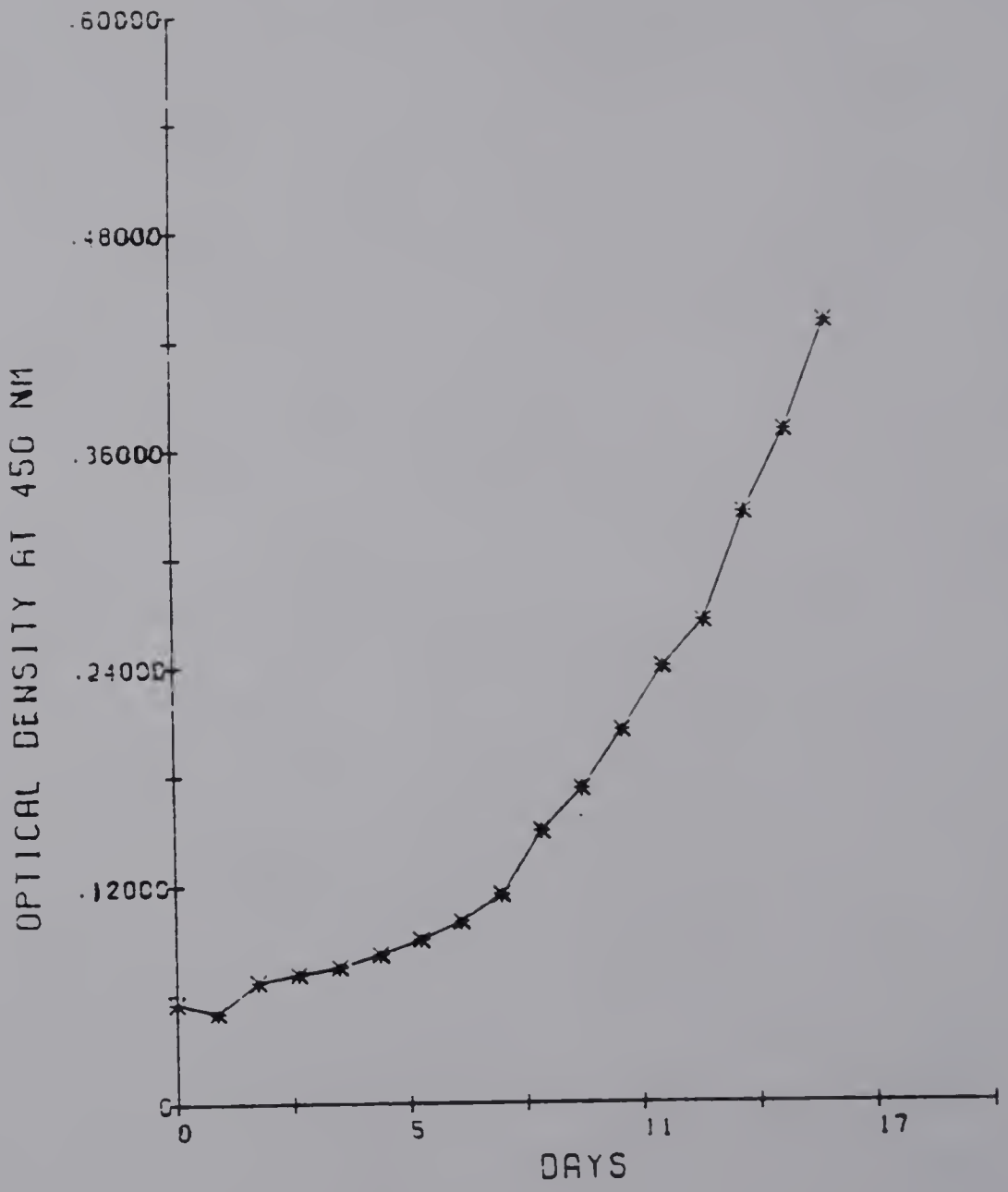

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 1b

Figure 2a

Growth curve A of Gloeothece ATCC 27152 cultivated on medium BG-11 without a combined nitrogen source.

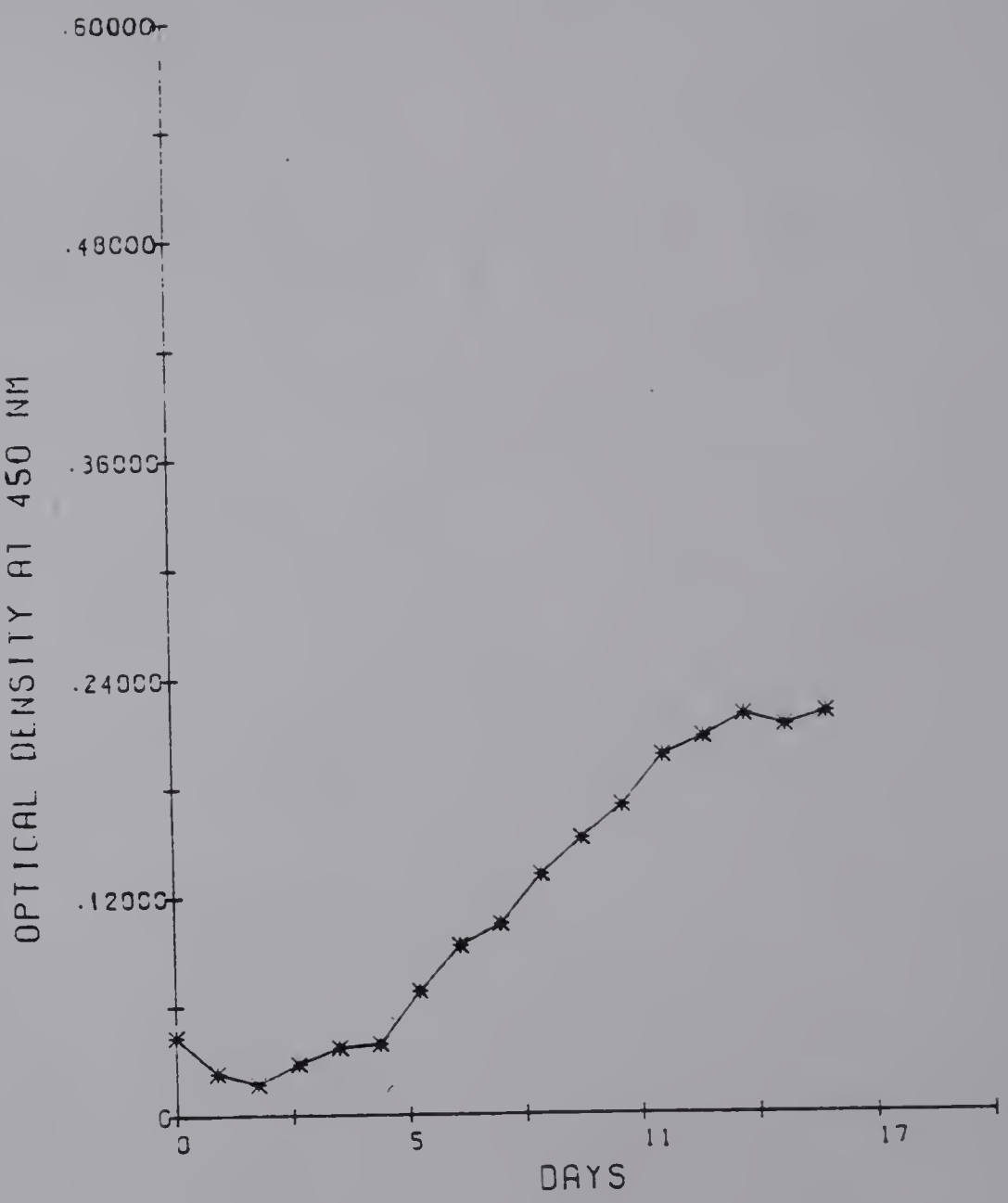

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 2a

Figure 2b

Growth curve B of Gloeothece ATCC 27152 cultivated on medium BG-11 without a combined nitrogen source.

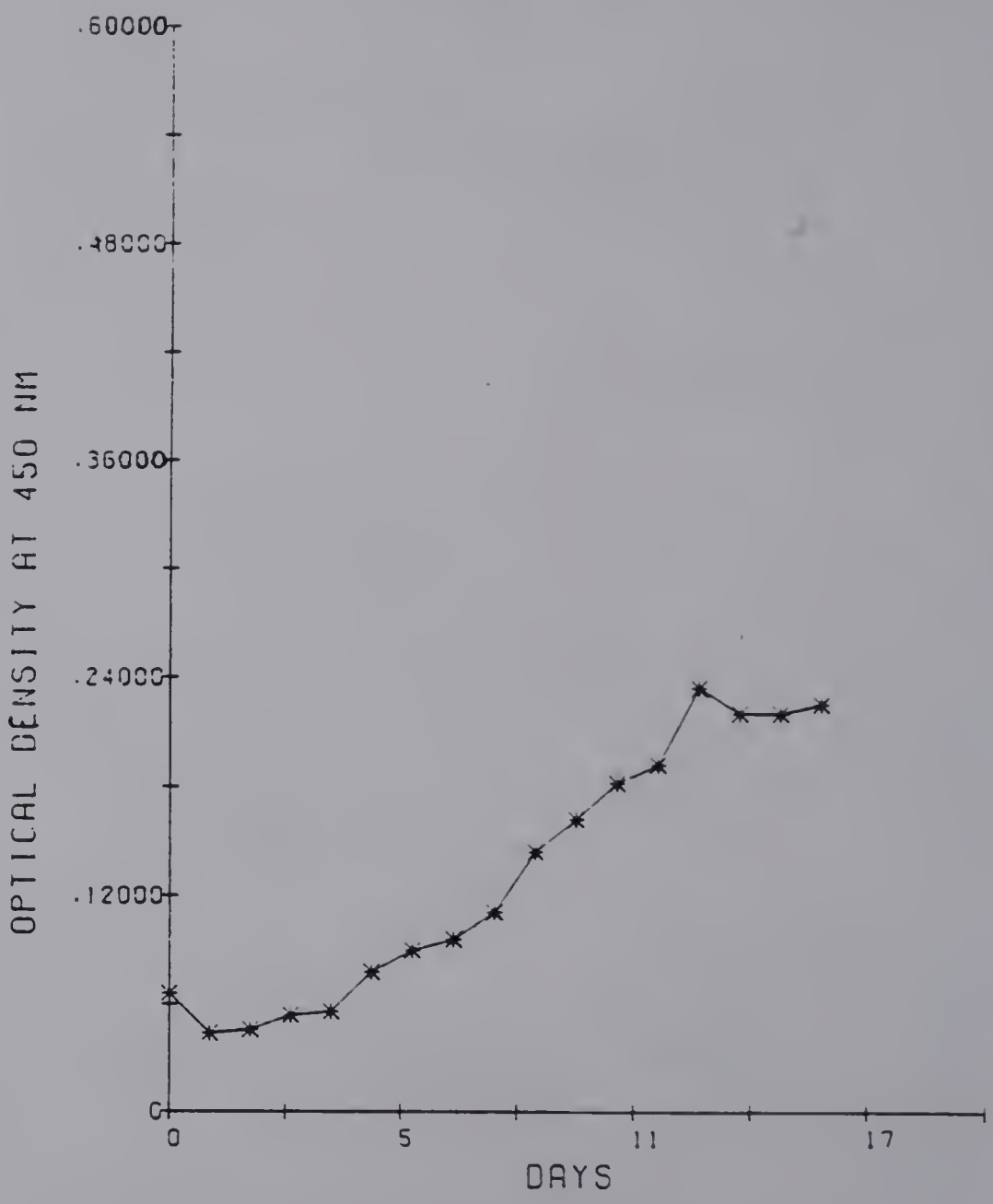

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 2b

Figure 2c

Growth curve C of Gloeothece ATCC 27152 cultivated on medium BG-11 without a combined nitrogen source.

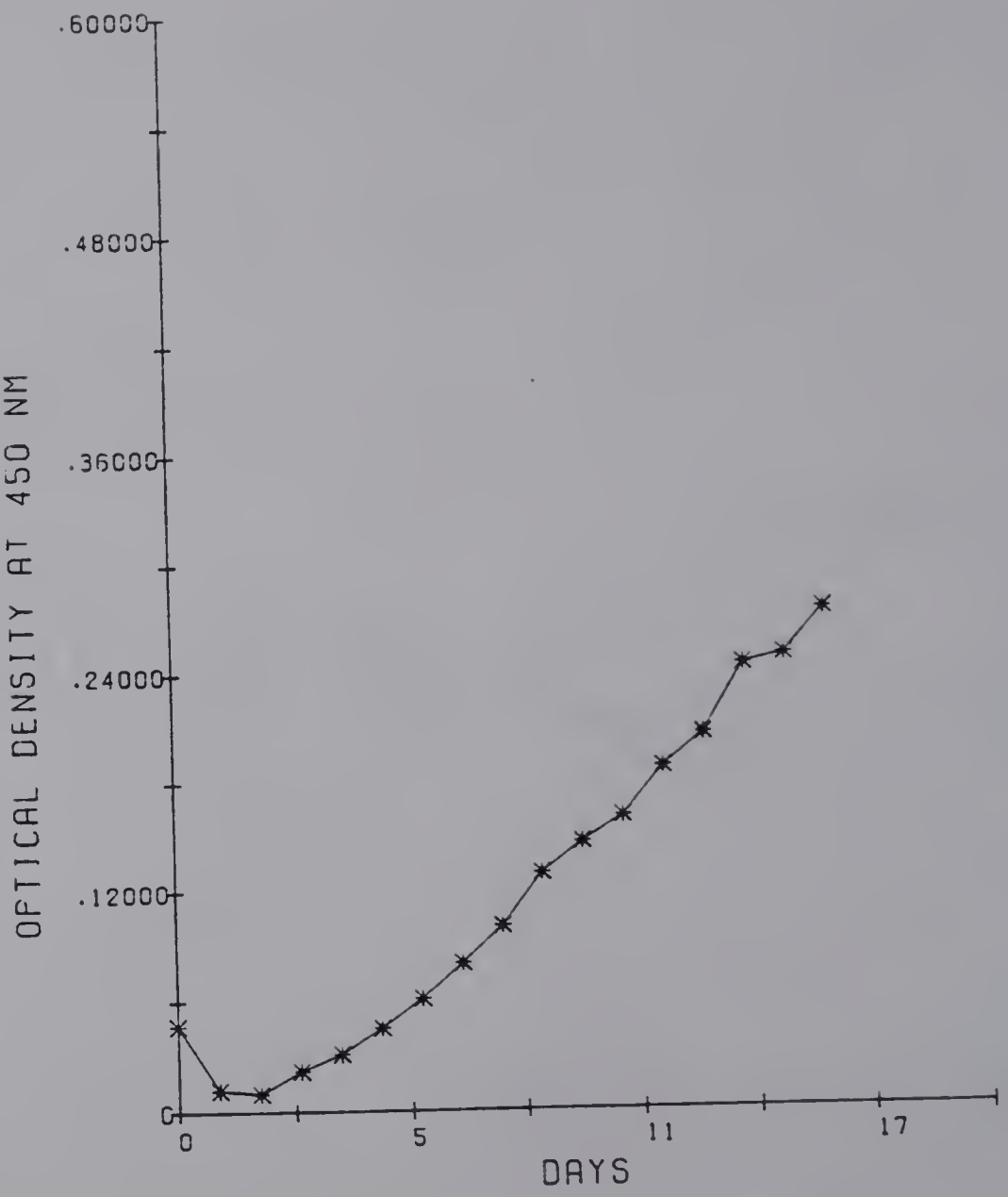

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 2c

Figure 3a

Growth curve A of Gloeothece ATCC 27152 cultivated on medium BG-11 supplemented with 100 mg/L of $NaHCO_3$.

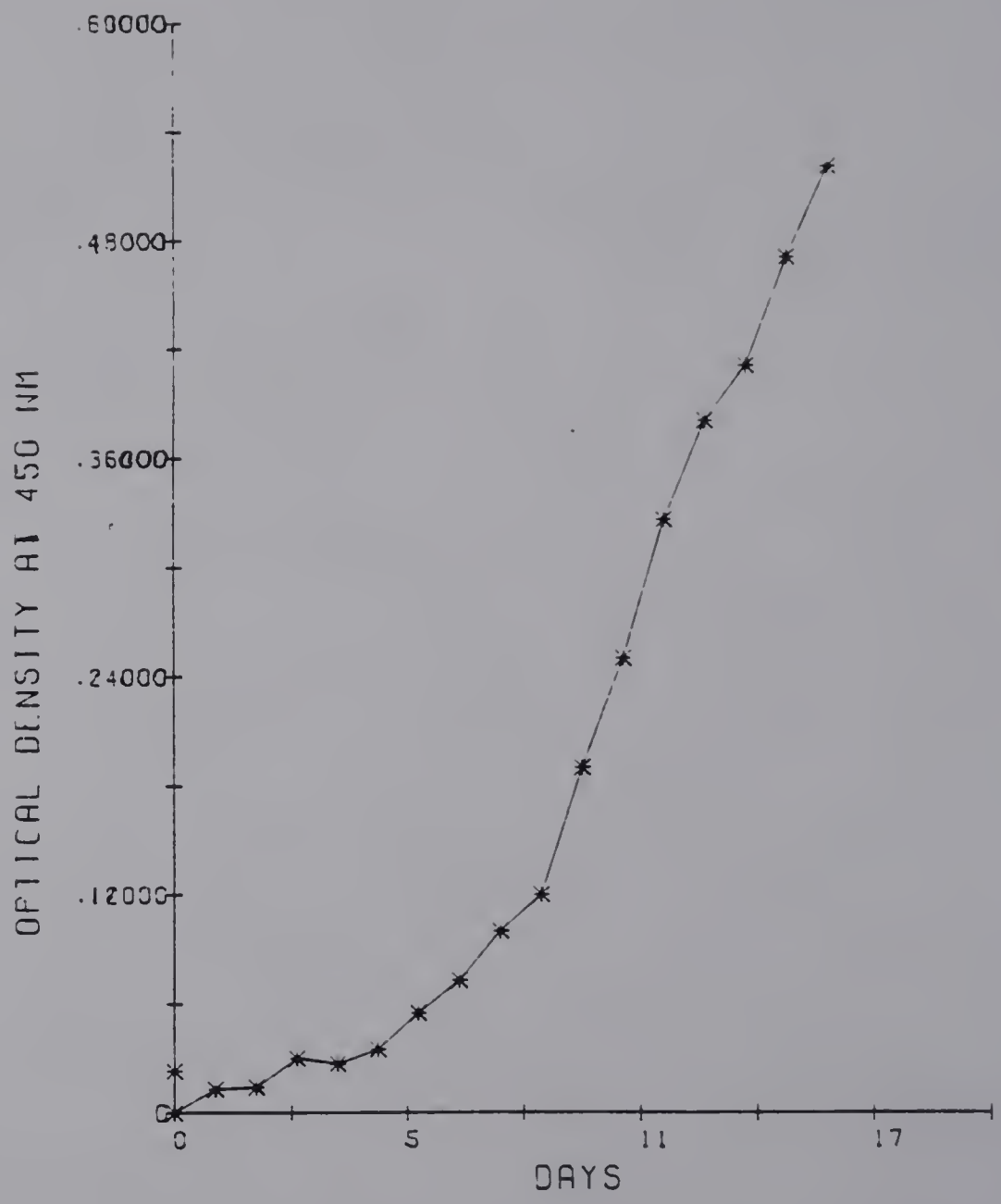

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 3a

Figure 3b

Growth curve B of Gloeothece ATCC 27152 cultivated on medium BG-11 supplemented with 100 mg/L of $NaHCO_3$.

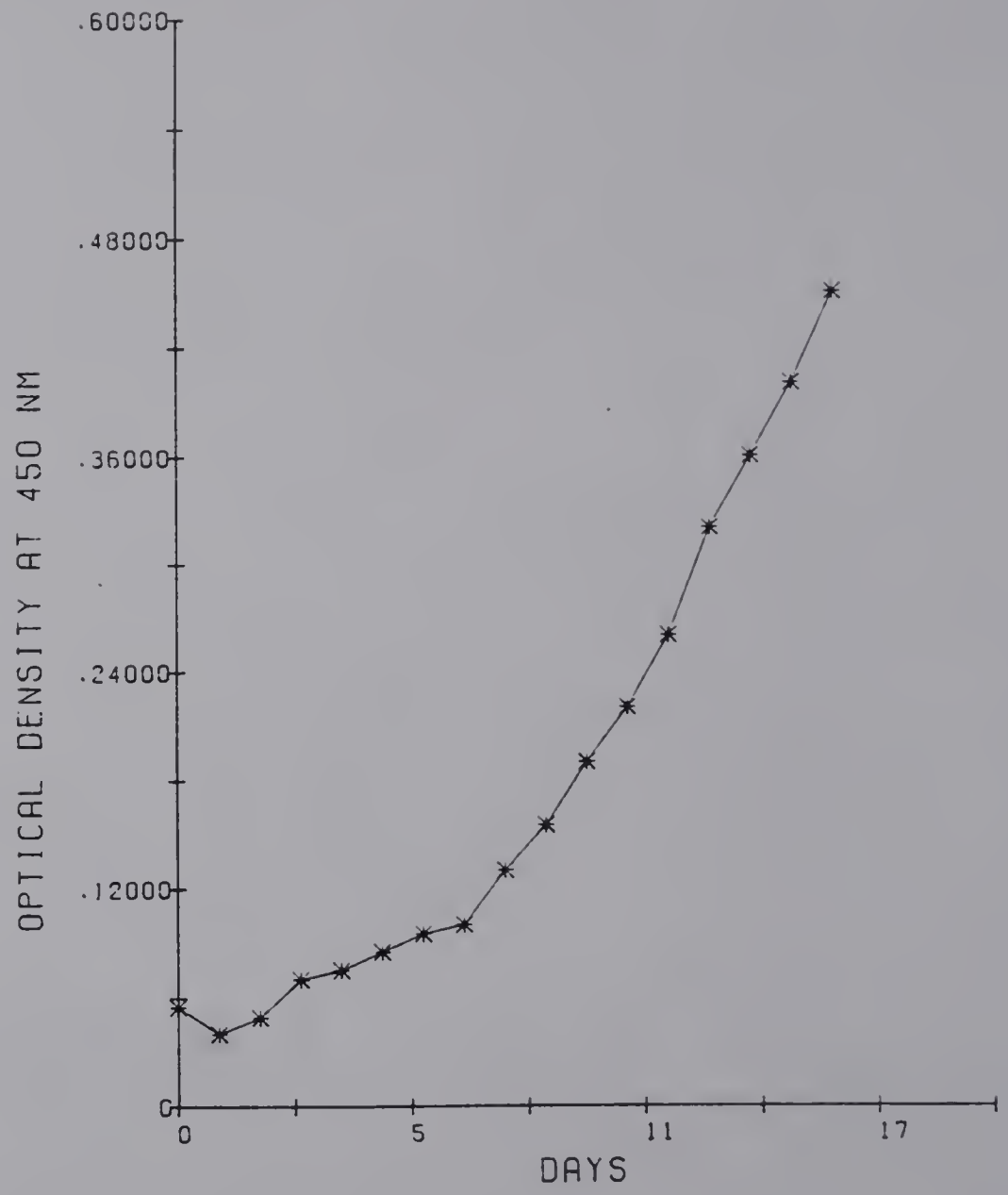

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 3b

Figure 3c

Growth curve C of Gloeothece ATCC 27152 cultivated on medium BG-11 supplemented with 100 mg/L of $NaHCO_3$.

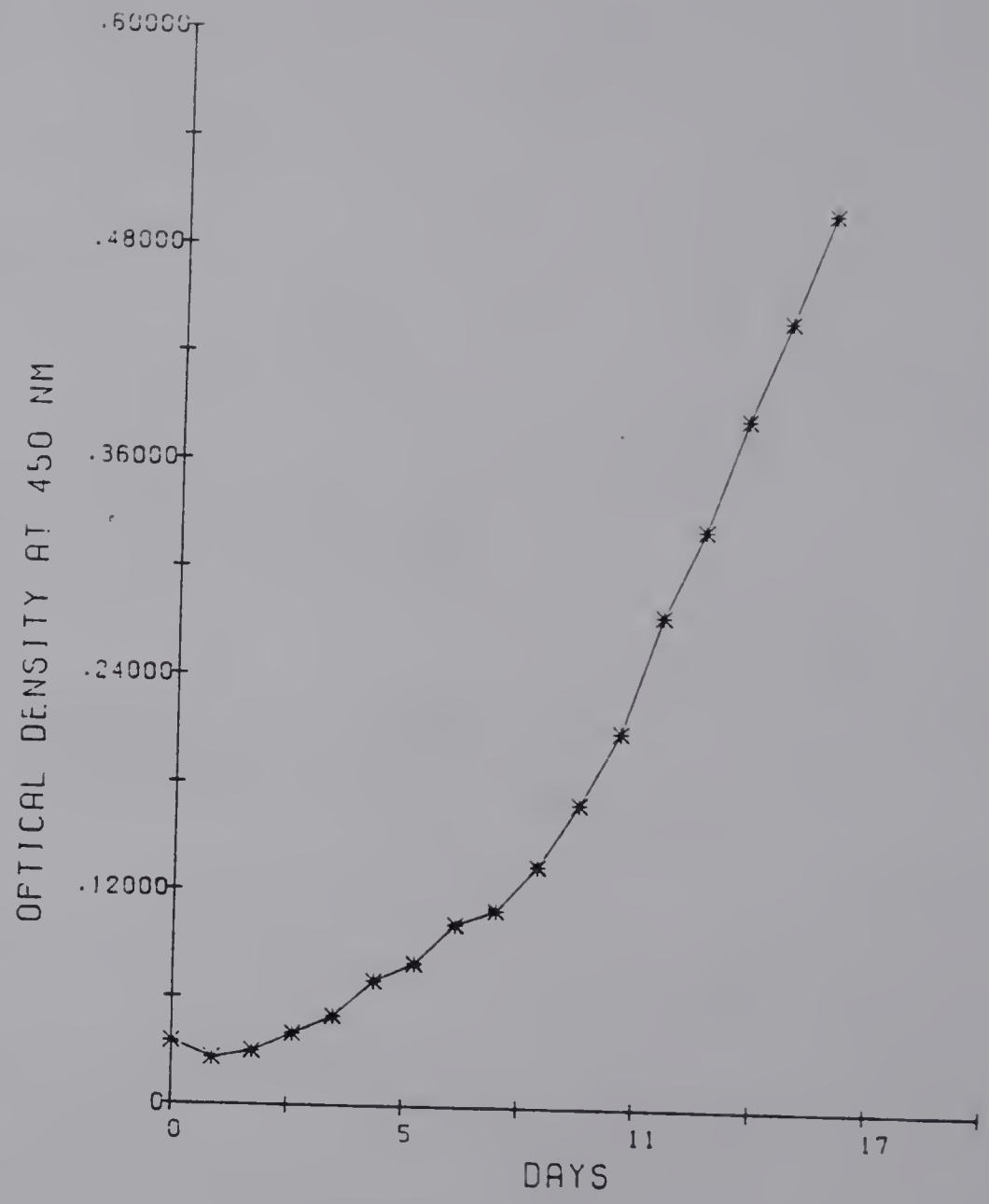

.60000
.48000
.36000
.24000
.12000
0
OPTICAL DENSITY AT 450 NM
0
5
11
17
DAYS

Figure 3c

FIGURE 4

The visible spectrum of the crude sheath preparation prior to high speed centrifugation.

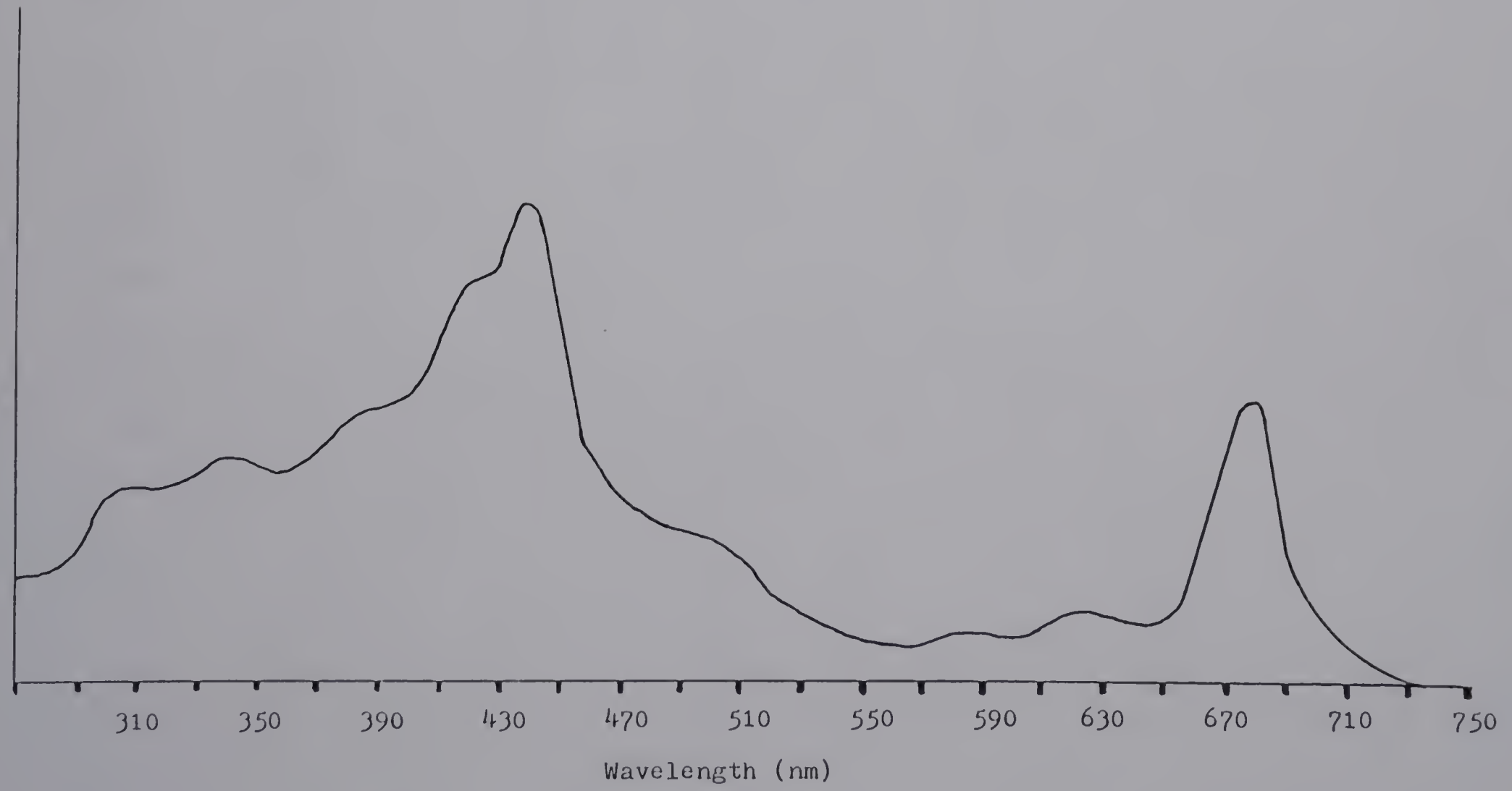

Relative Response
310
350
390
430
470
510
550
590
630
670
710
750
Wavelength (nm)

Figure 4

FIGURE 5

The visible spectrum of the phycobilin pigments contained in the aqueous phase after centrifugation of the crude sheath preparation at 38,000g for 10 minutes.

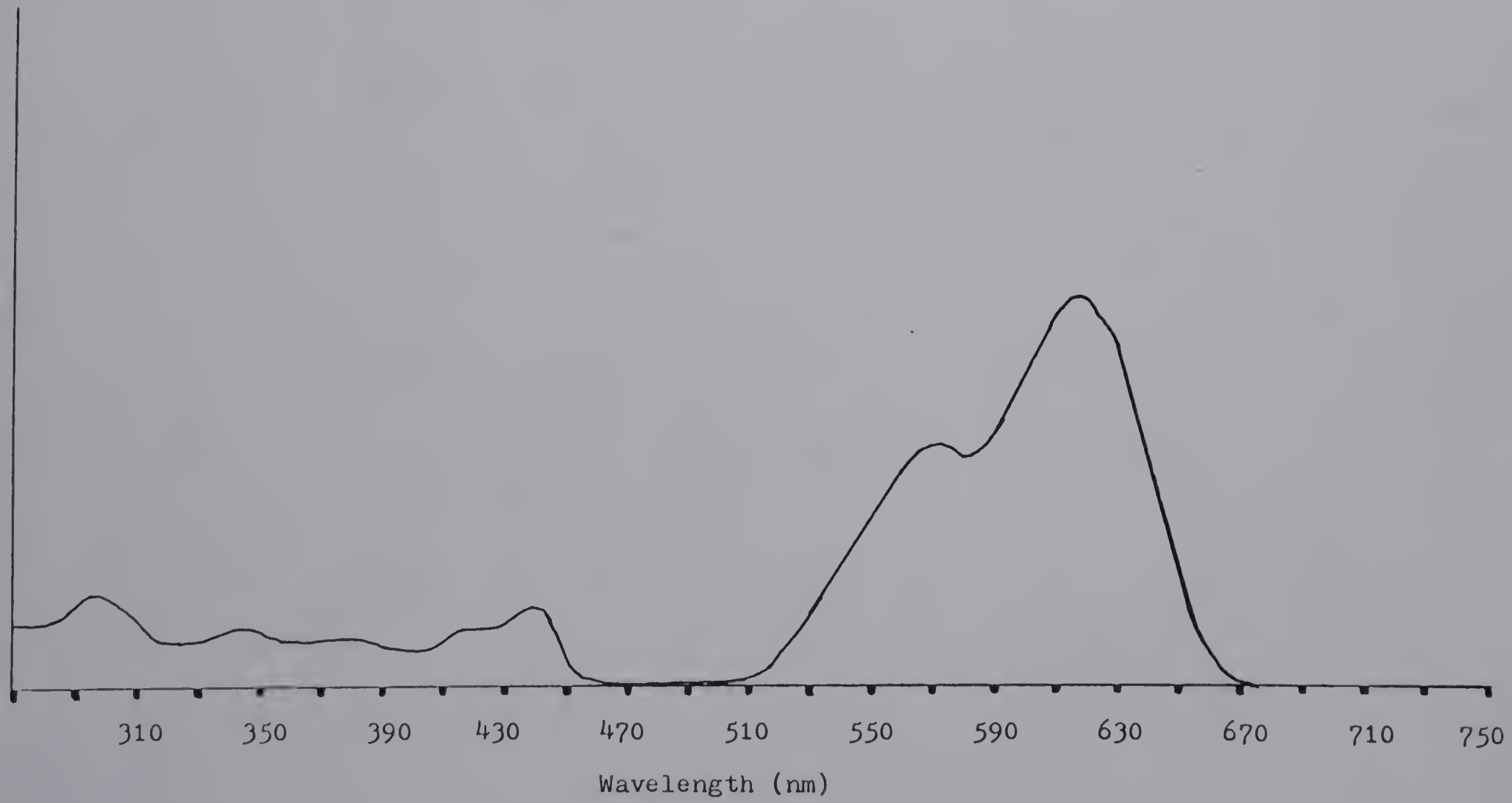

Relative Response
310
350
390
430
470
510
550
590
630
670
710
750
Wavelength (nm)

Figure 5

Figure 6

Amino acid standards containing 100nM of the following: Asp (1), Thr (2), Ser (3), Glut (4), Pro (5), 1/2 Cyst (6), Gly (7), Ala (8), Val (9), Meth (10), Isoleu (11), Leu (12), norLeu (13), Tyr (14), Phenala (15), Hist (16), Lys (17), Tryp (18a) and Arg (19). Peak 18 represents the ammonium ion liberated from the basic amino acids. 2/7/85

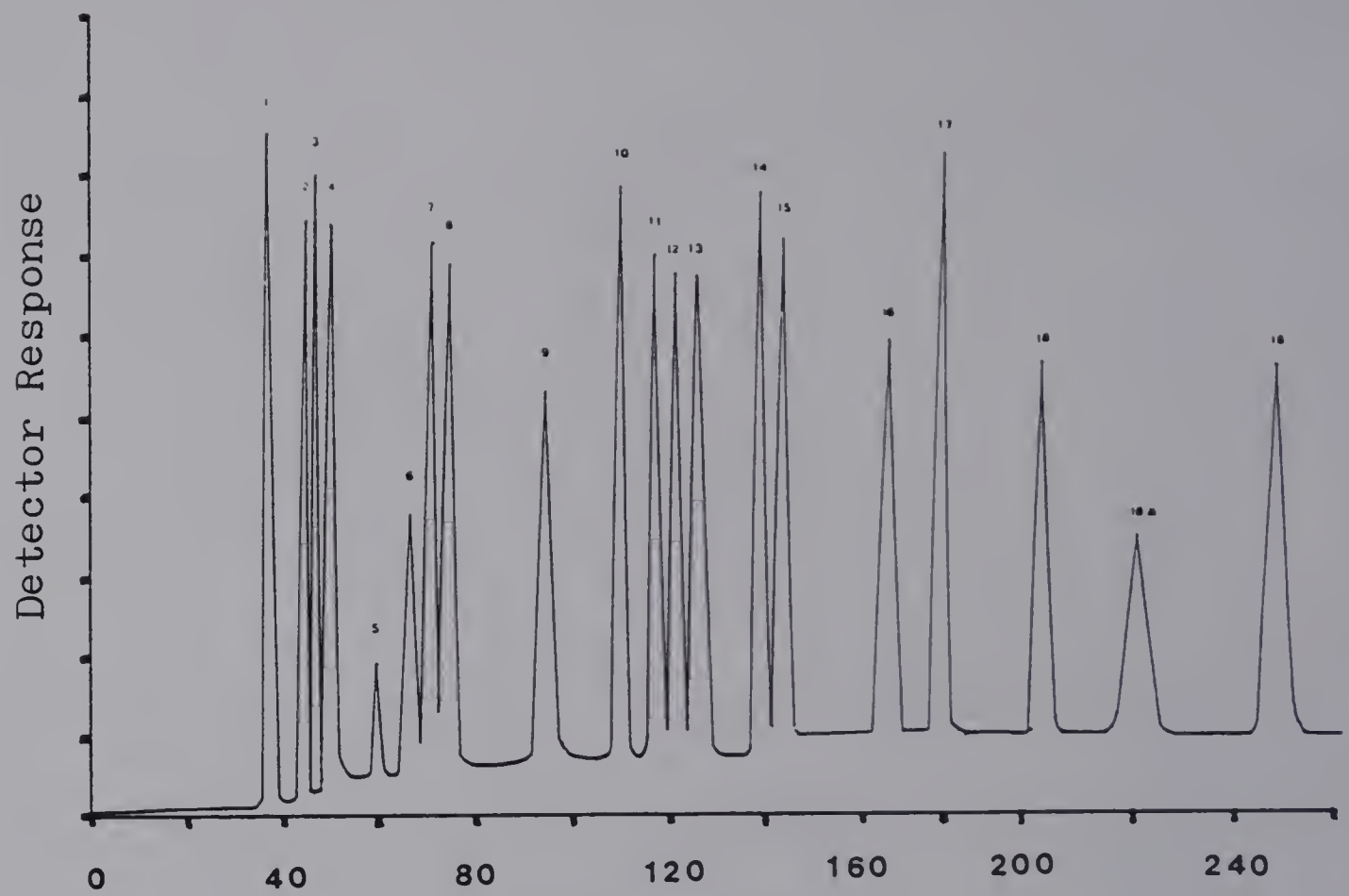

Detector Response
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
18A
19
0
40
80
120
160
200
240
Time (minutes)

Figure 6

Figure 7

Amino acid composition of a 10.0mg FDS-G-NO_3 sample hydrolyzed for 20 hours at 110 C in 6 N HCl. 2/7/85

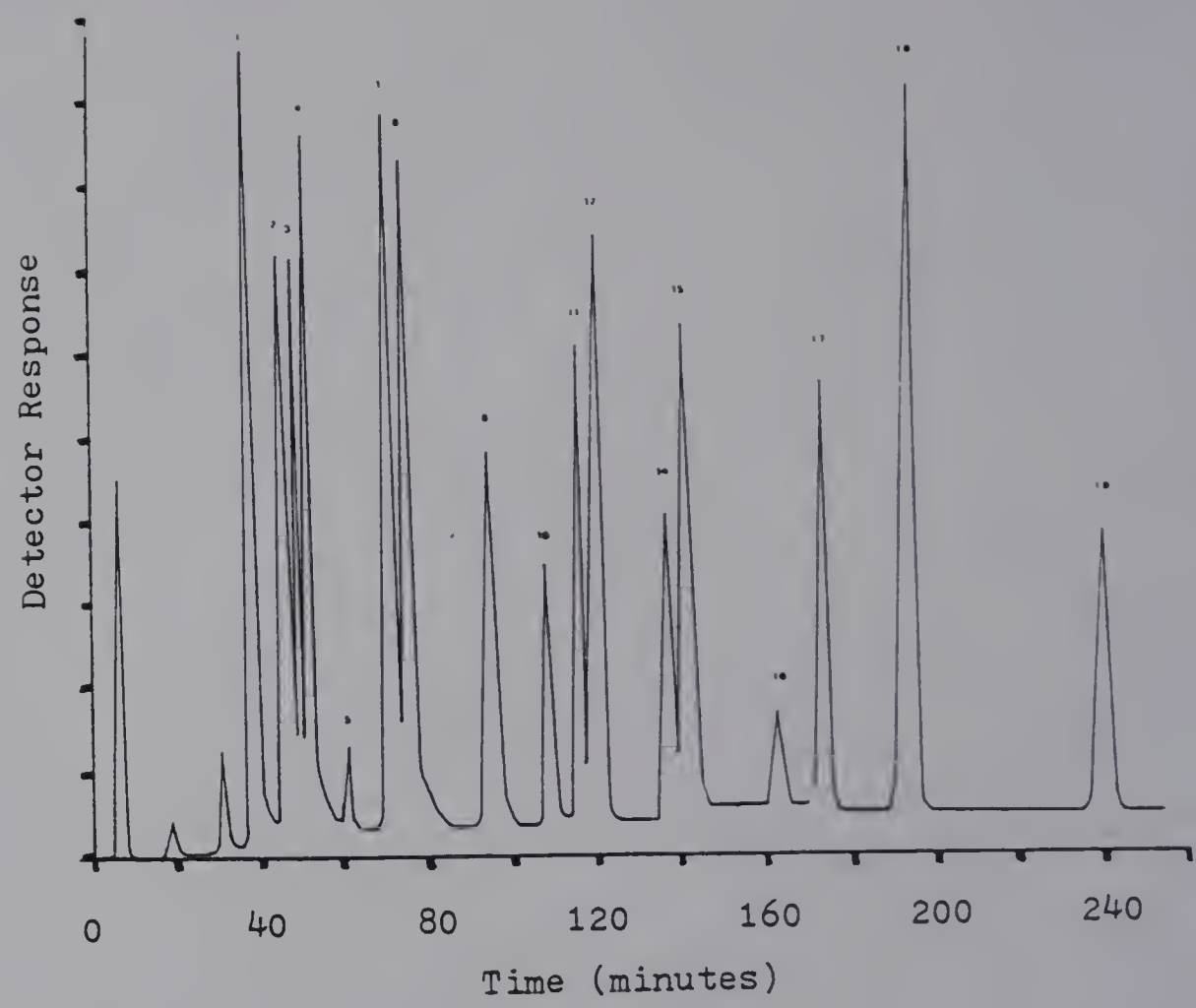


Figure 7

Figure 8

Amino acid composition of a 20.0mg FDS-SDS-NO_3 sample hydrolyzed for 20 hours at 110 C in 6 N HCl. 2/26/85

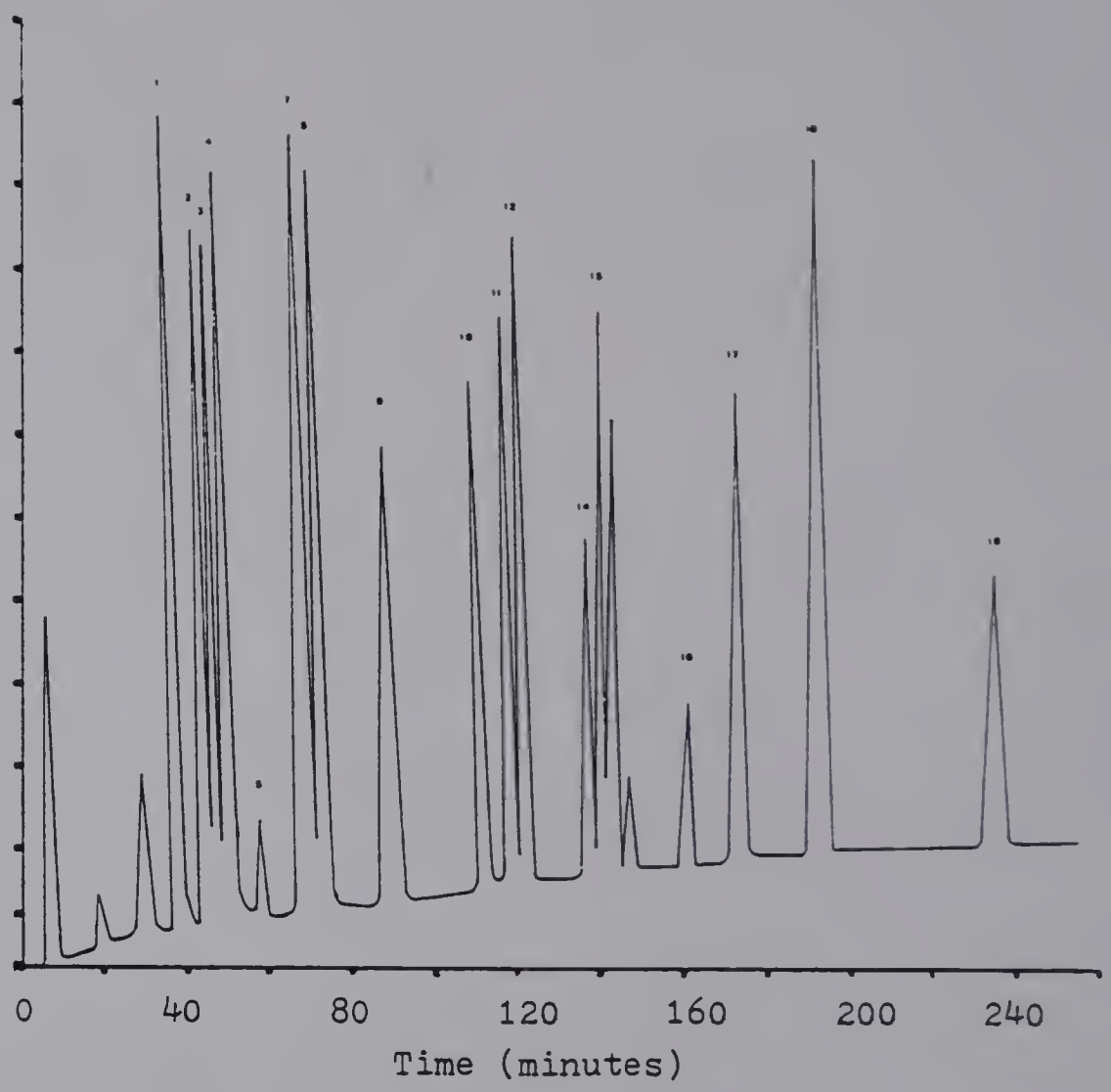


Figure 8

Figure 9

Amino acid standards containing 100nM of the following: Asp (1), Thr (2), Ser (3), Glut (4), Pro (5), 1/2 Cyst (6), Gly (7), Ala (8), Val (9), Meth (10), Isoleu (11), Leu (12), norLeu (13), Tyr (14), Phenala (15), Hist (16), Lys (17), Tryp (18a) and Arg (19). Peak 18 represents the ammonium ion liberated from the basic amino acids. 7/19/85

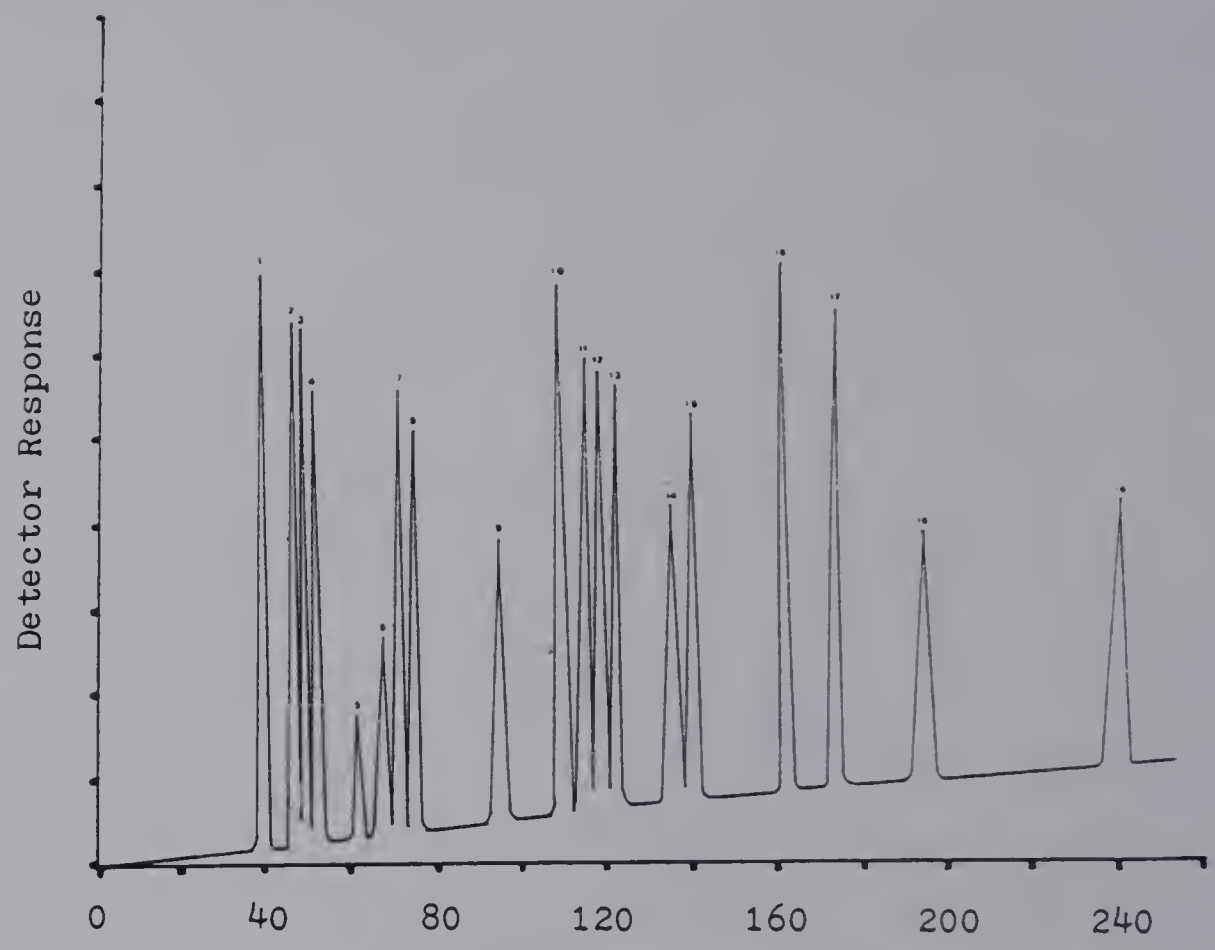


Figure 9

Figure 10

Amino acid composition of a 4.87mg FDS-G-NO_3 sample hydrolyzed for 20 hours at 110 C in 6 N HCl. 7/19/85

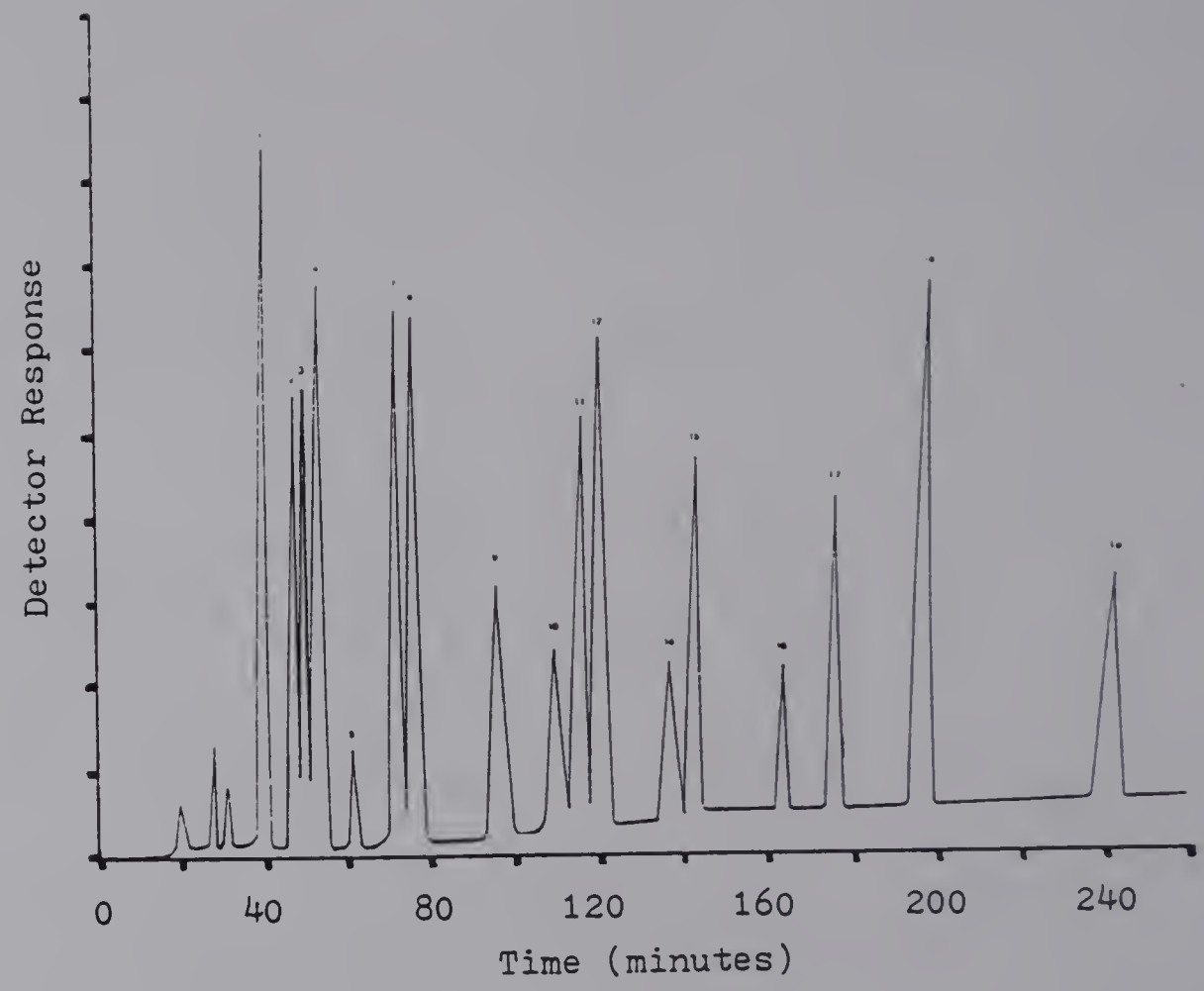


Figure 10

Figure 11

Amino acid composition of a 4.97mg FDS-W-NO_3 sample hydrolyzed for 20 hours at 110 C in 6 N HCl. 7/19/85

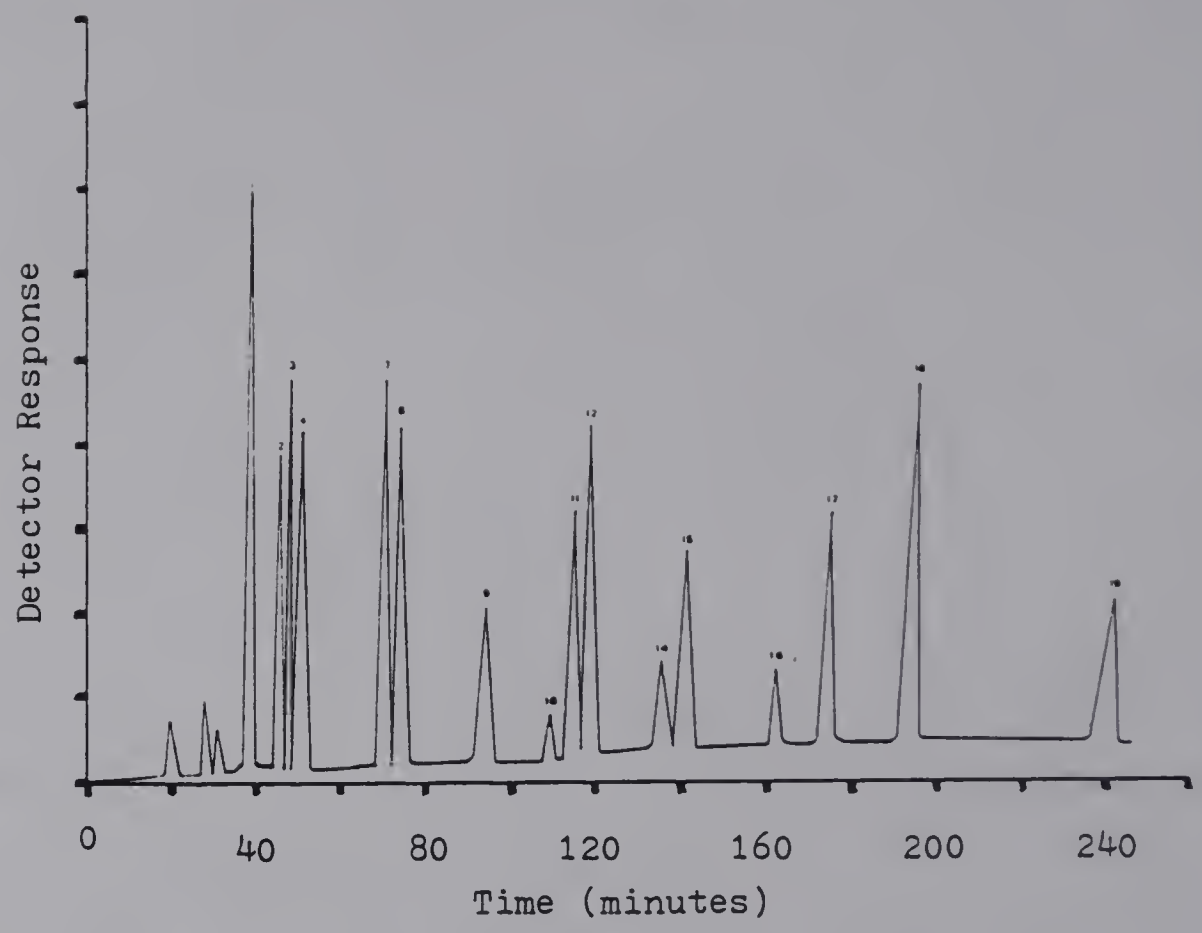


Figure 11

Figure 12

Photograph of a SDS-PAGE gel exhibiting the protein bands from two samples of FDS-G-no NO_3 material ineffectively treated with lysozyme and SDS (A=4.98mg, B=5.00mg). Wells number 1 and 3 contain 10ul of sample extract while wells 2 and 4 contain 20ul. Protein standards represent the following molecular weights: 200,000, 116,250, 92,500, 66,200, and 45,000.

A B

1 2 3 4

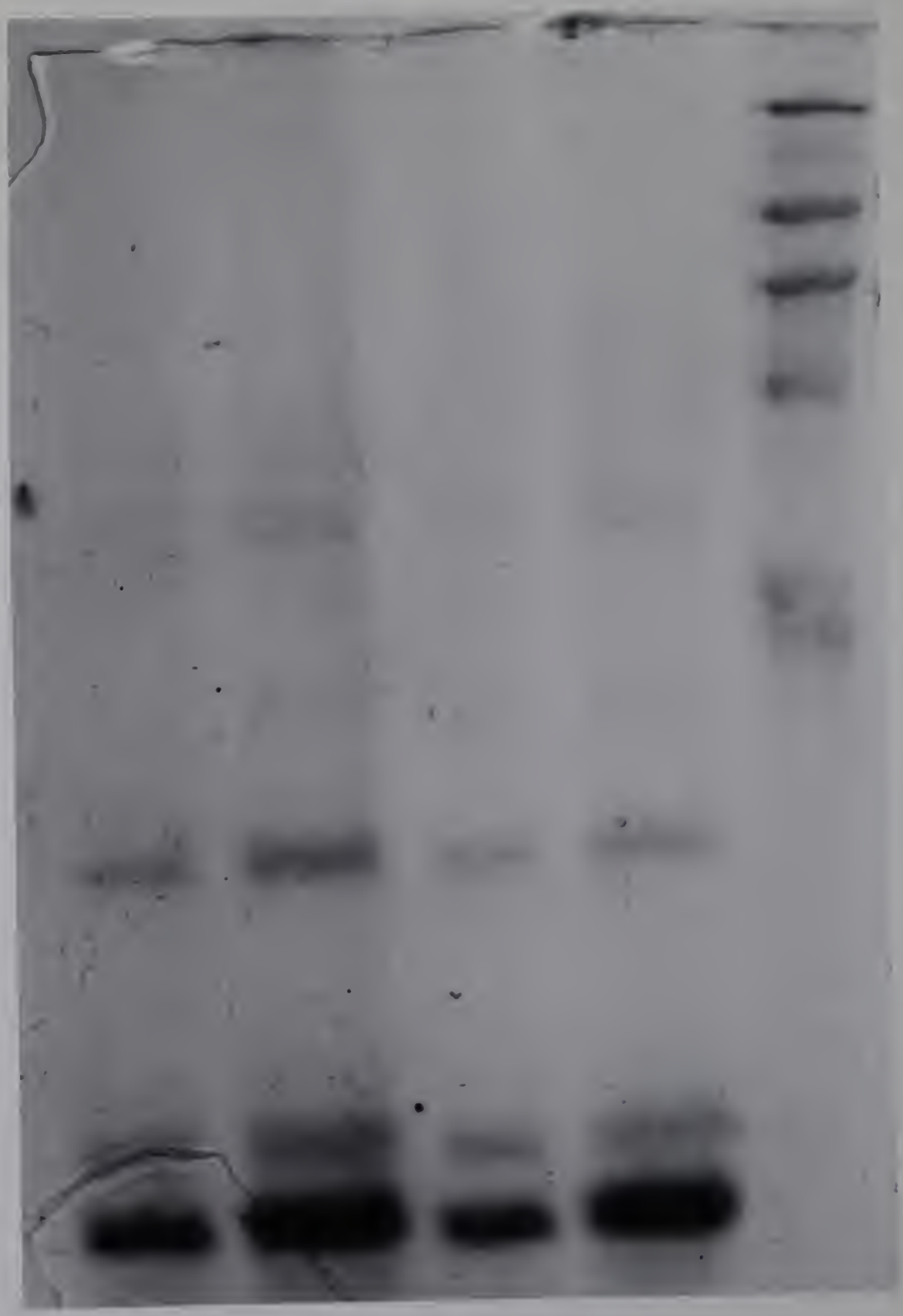


Figure 12

Figure 13

Photograph of a SDS-PAGE gel exhibiting the protein bands from two samples of FDS-G-no NO_3 material (A=4.98mg of material properly treated with lysozyme and SDS , B=5.00mg of material ineffectively treated and re-extracted with sample buffer). Both wells received 30ul of sample extract. Protein standards represent the following molecular weights: 200,000, 97,400, 68,000, 43,000, 25,700, 18,400 and 14,300.

A B

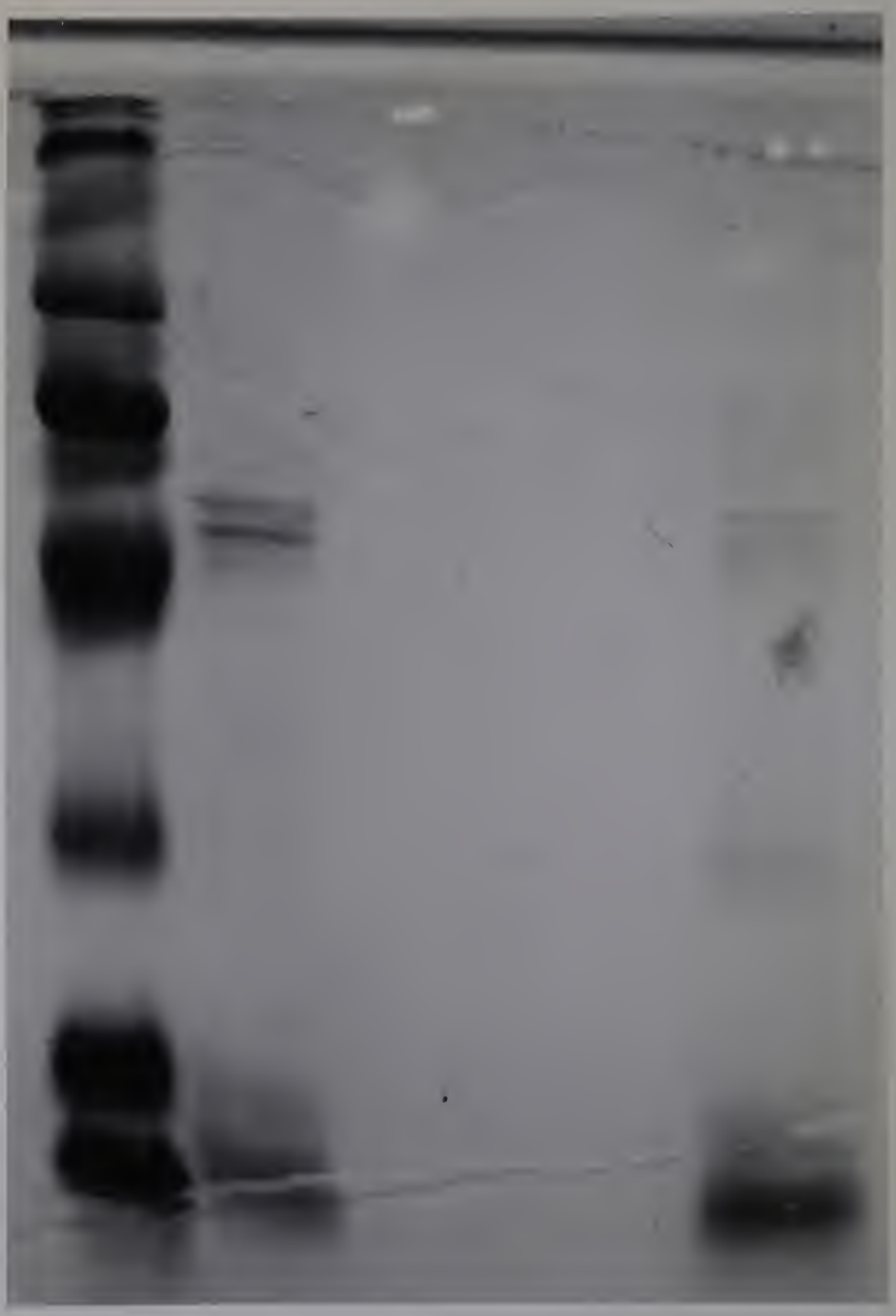
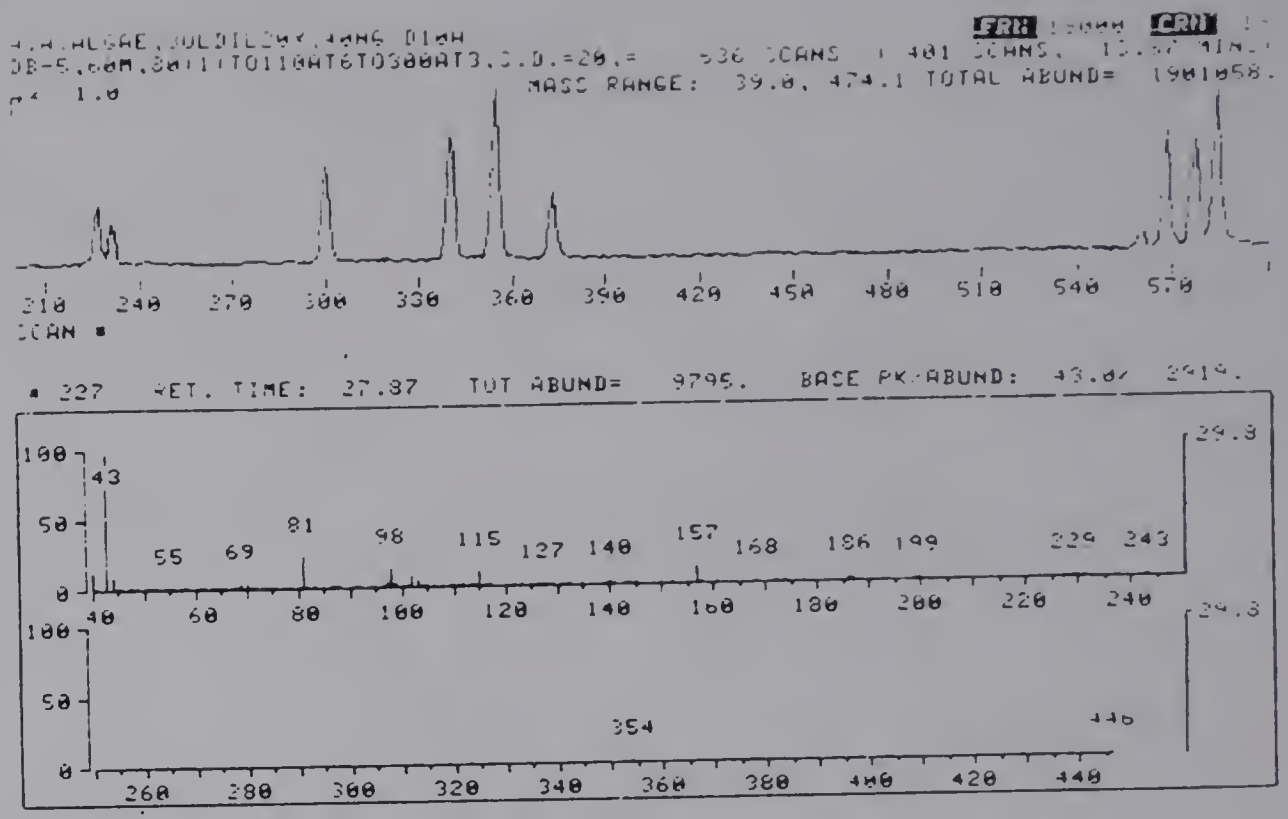


Figure 13

Figure 14A & B

Electron impact mass spectra of (A) an unknown component and (B) the internal GC-MS standard dioxane from the alditol acetate derivatization of FDS-W-no NO_3, hydrolyzed for 1 hour at 100 C in 2 N TFA.

A



B

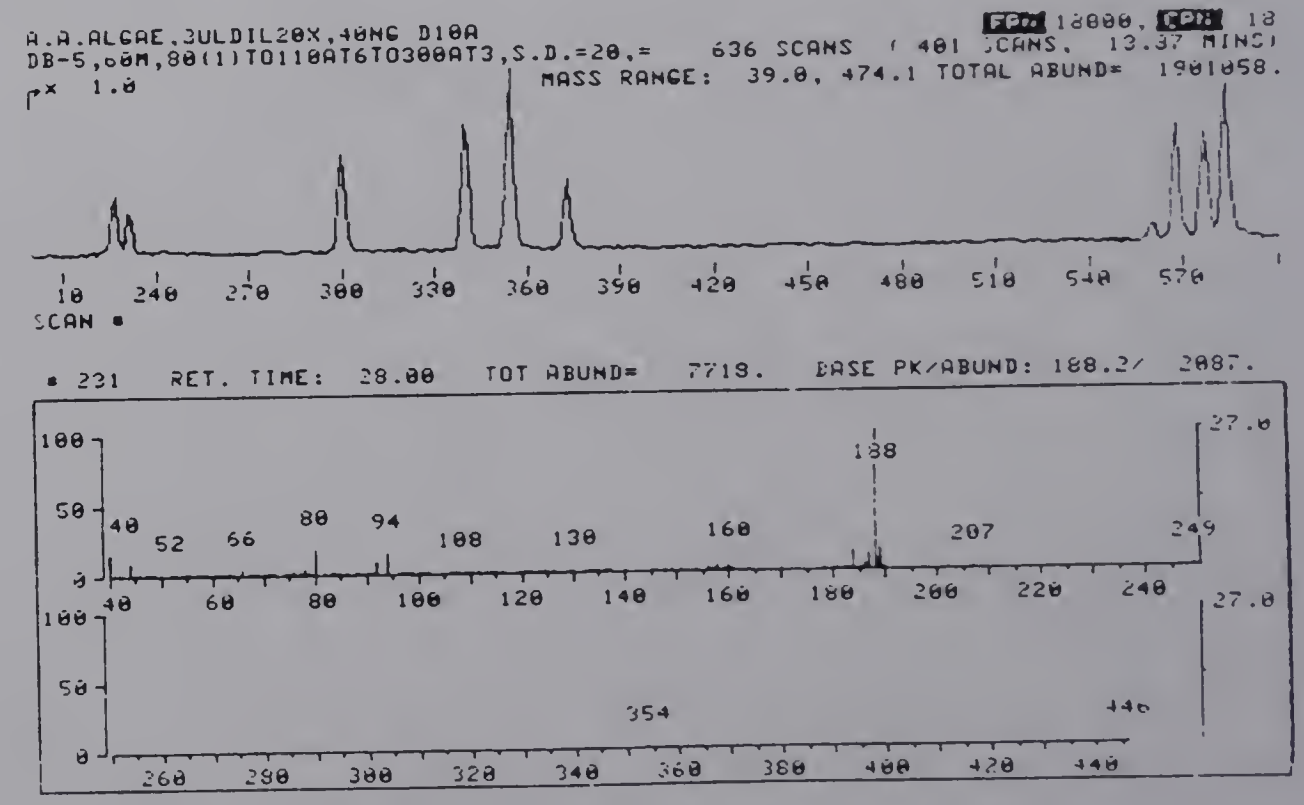
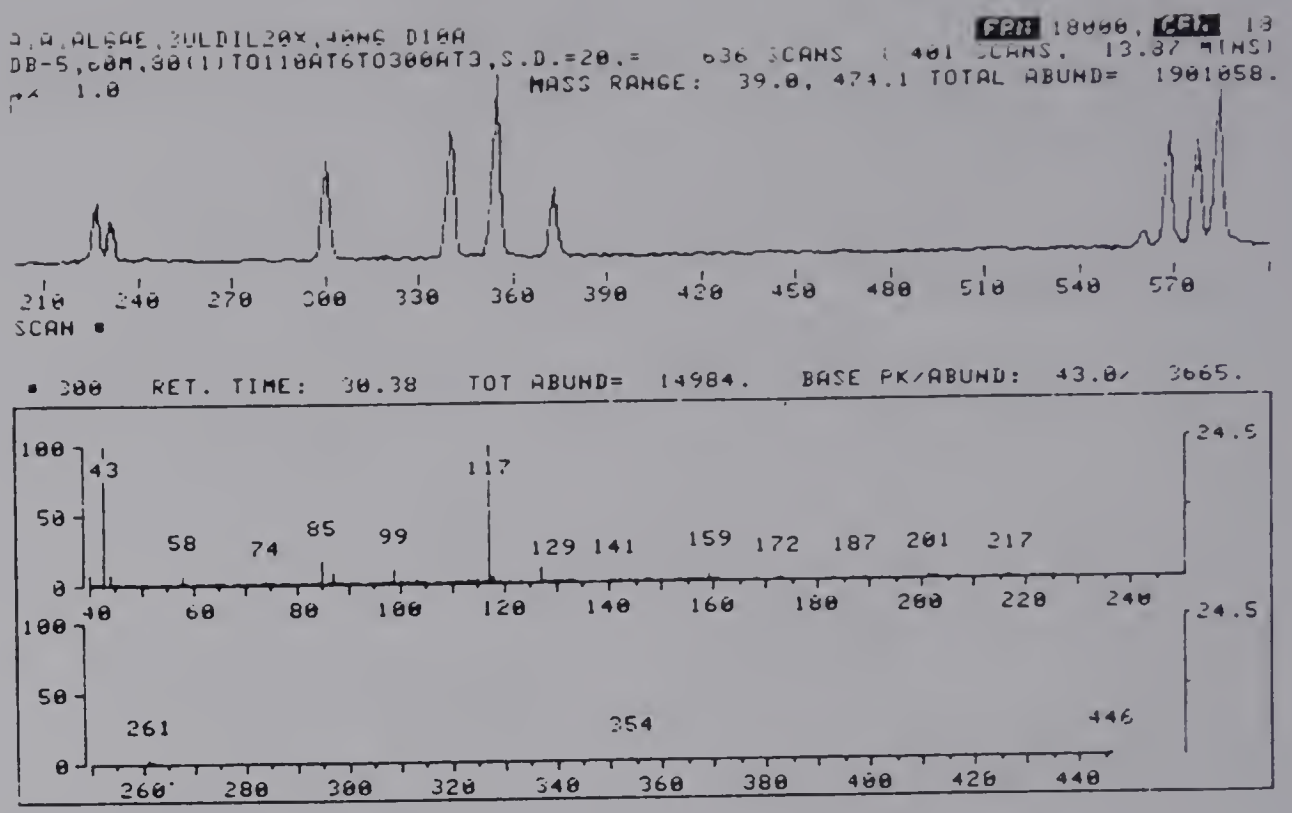


Figure 14 A & B

Figure 14C & D

Electron impact mass spectra of (C) 2-O-methyl xylose and (D) rhamnose from the alditol acetate derivatization of FDS-W-no NO_3, hydrolyzed for 1 hour at 100 C in 2 N TFA.

C



D

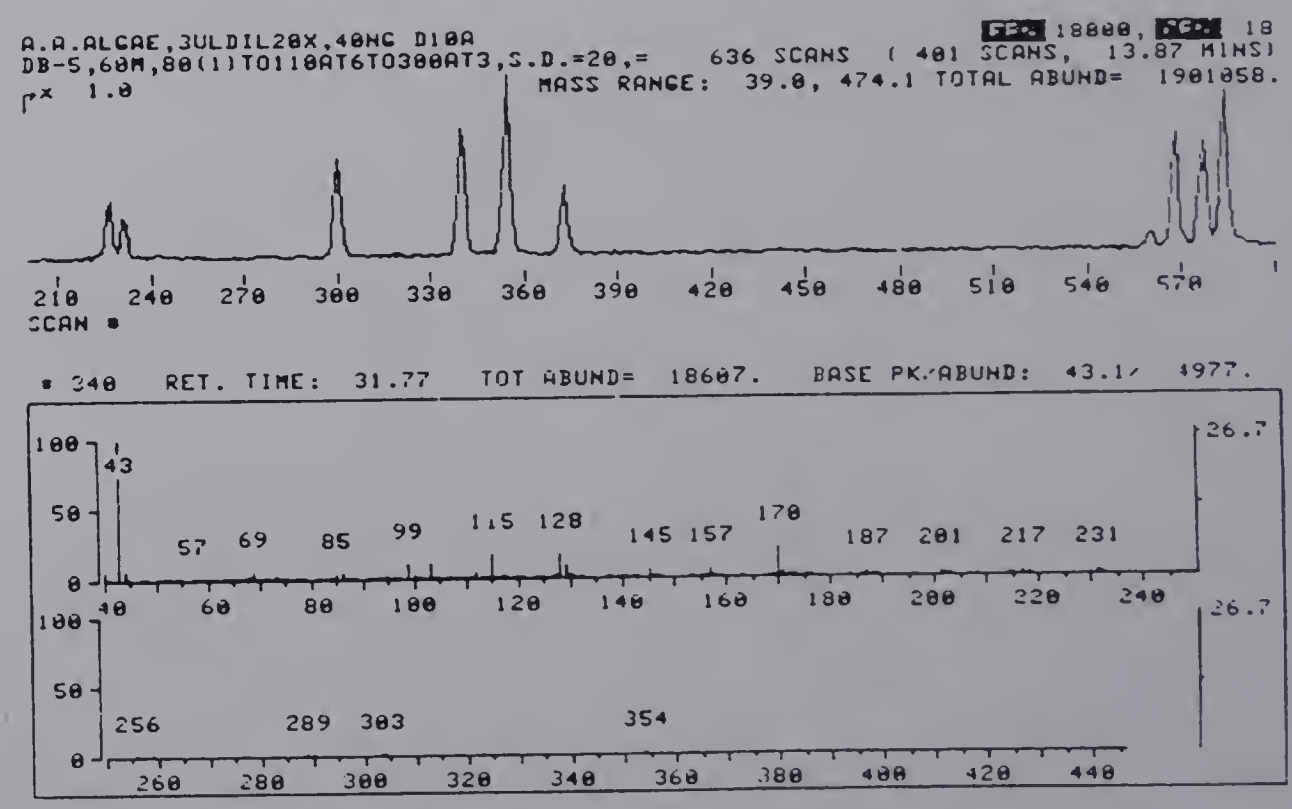
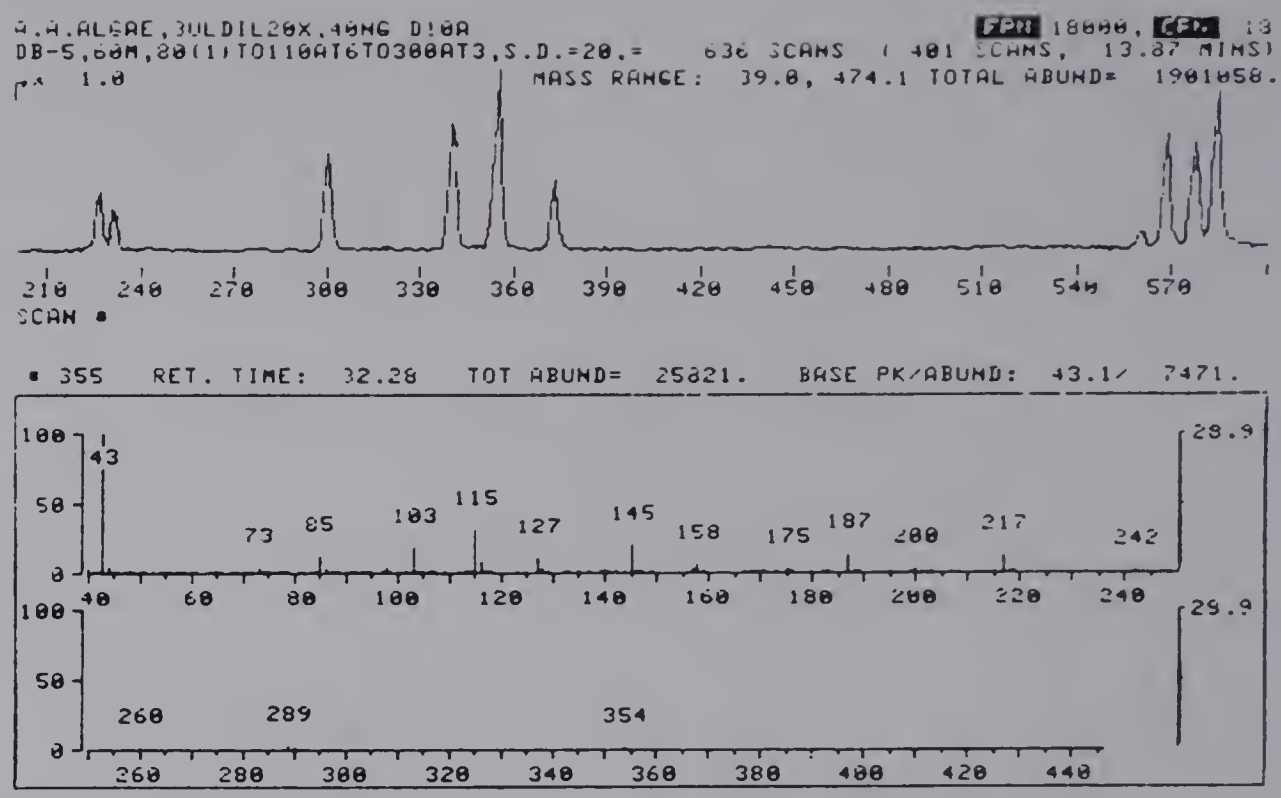


Figure 14C & D

Figure 14E & F

Electron impact mass spectra of (E) arabinose and (F) xylose from the alditol acetate derivatization of FDS-W-no NO_3, hydrolyzed for 1 hour at 100 C in 2 N TFA.

E



F

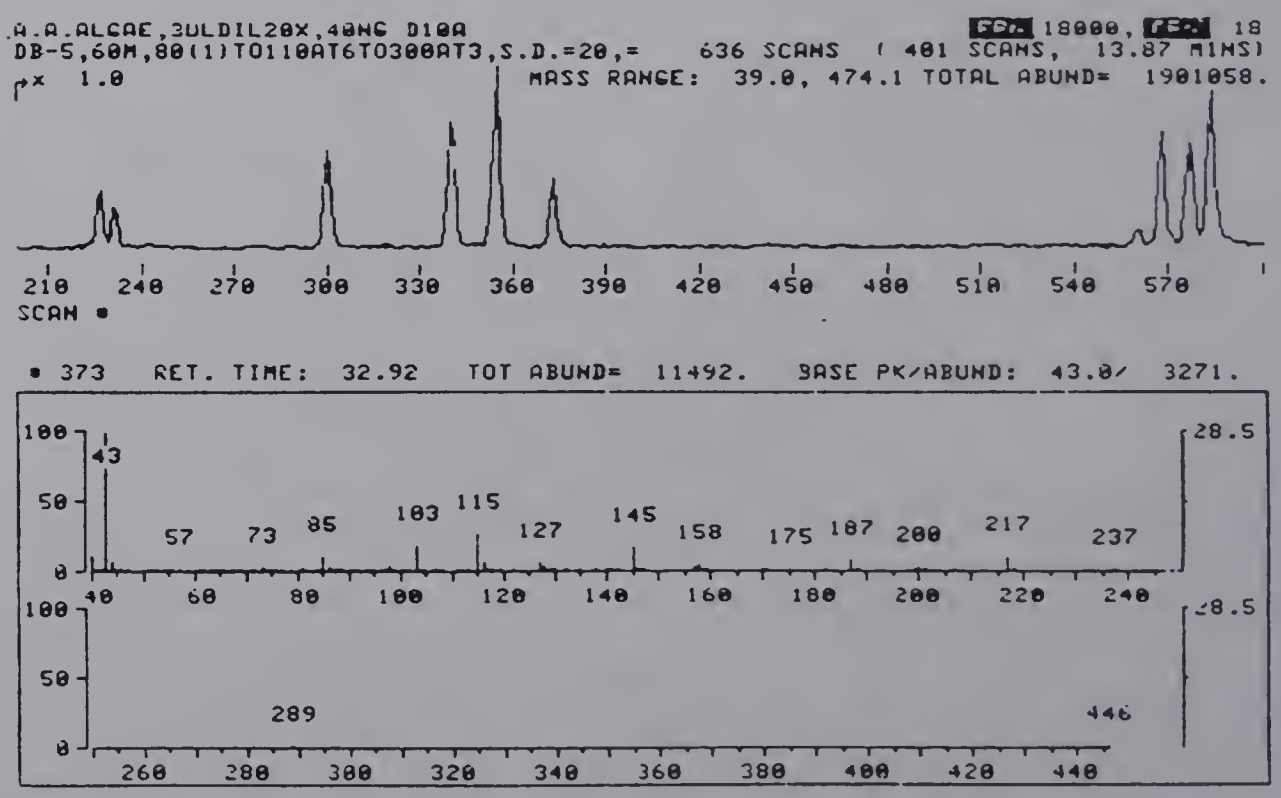
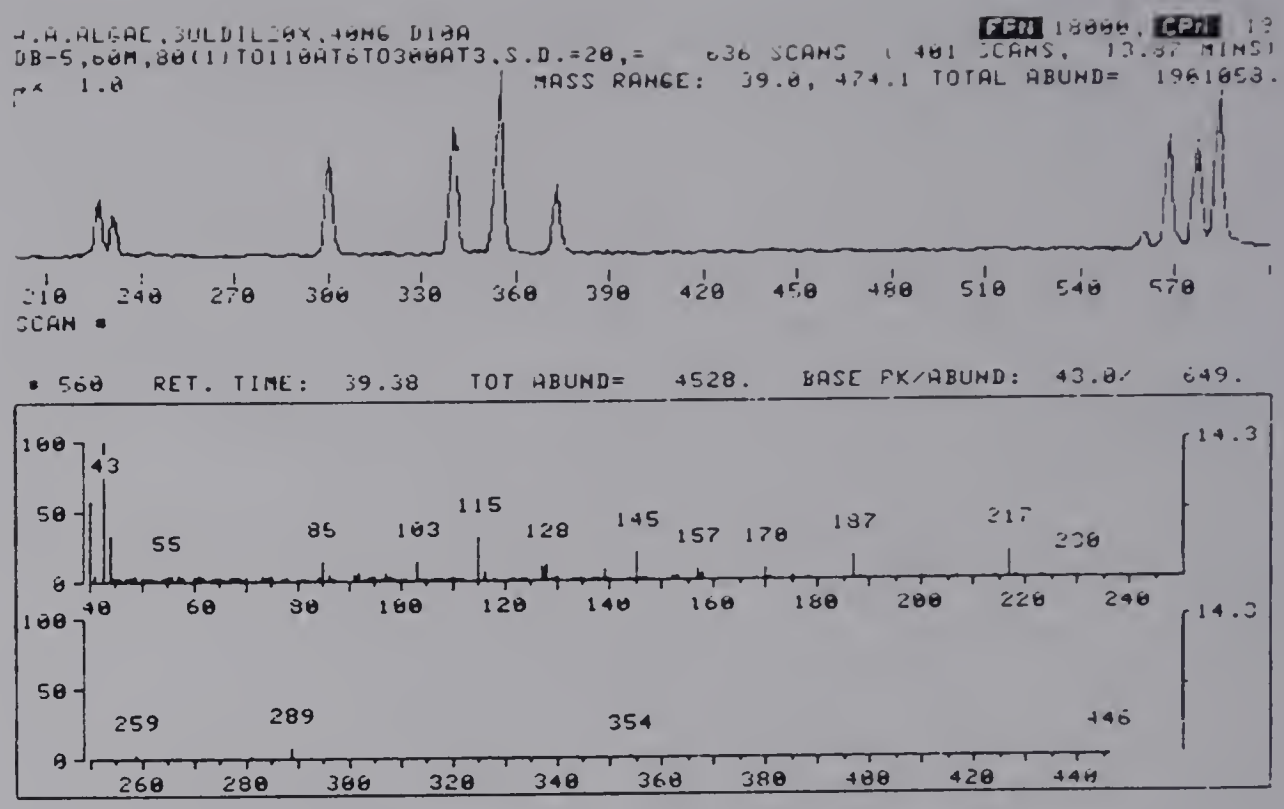


Figure 14E & F

Figure 14G & H

Electron impact mass spectra of (G) an unknown component and (H) mannose from the alditol acetate derivatization of FDS-W-no NO_3, hydrolyzed for 1 hour at 100 C in 2 N TFA.

G



H

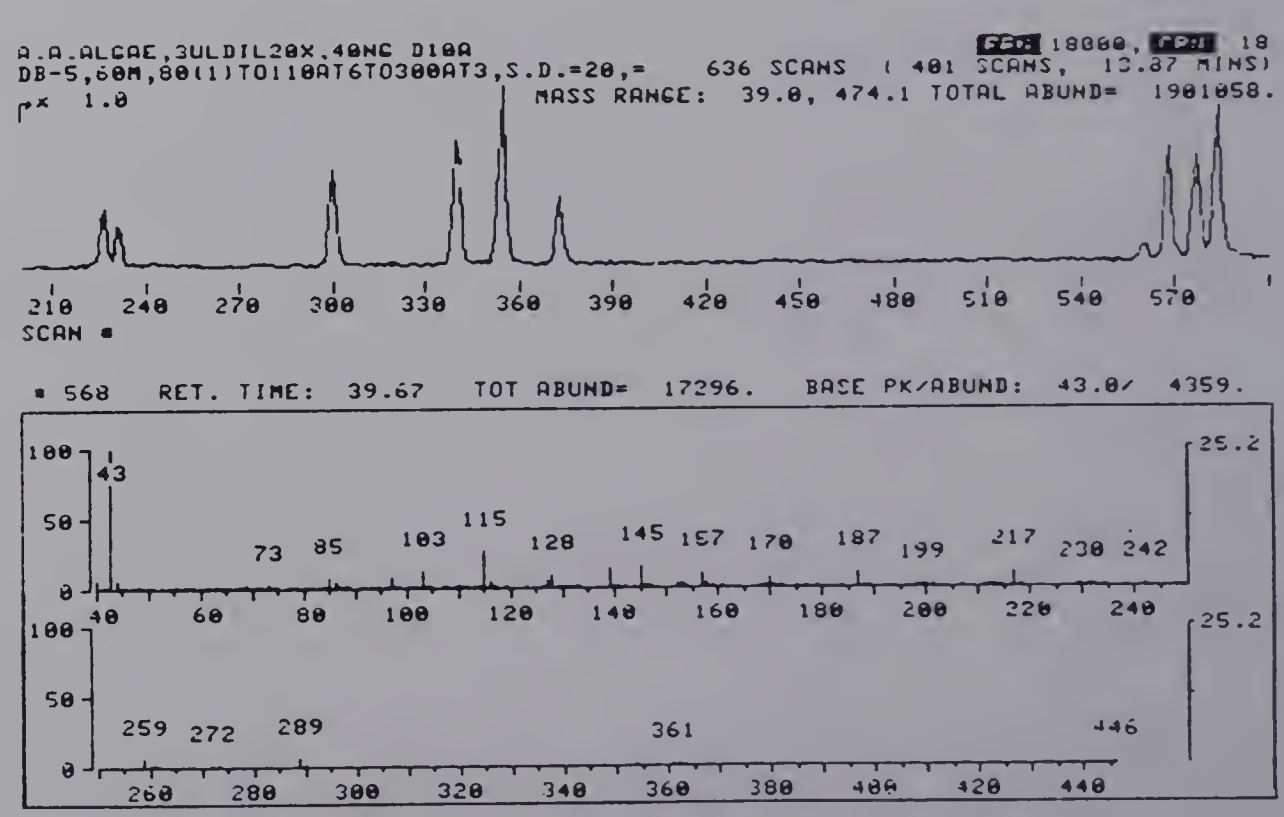
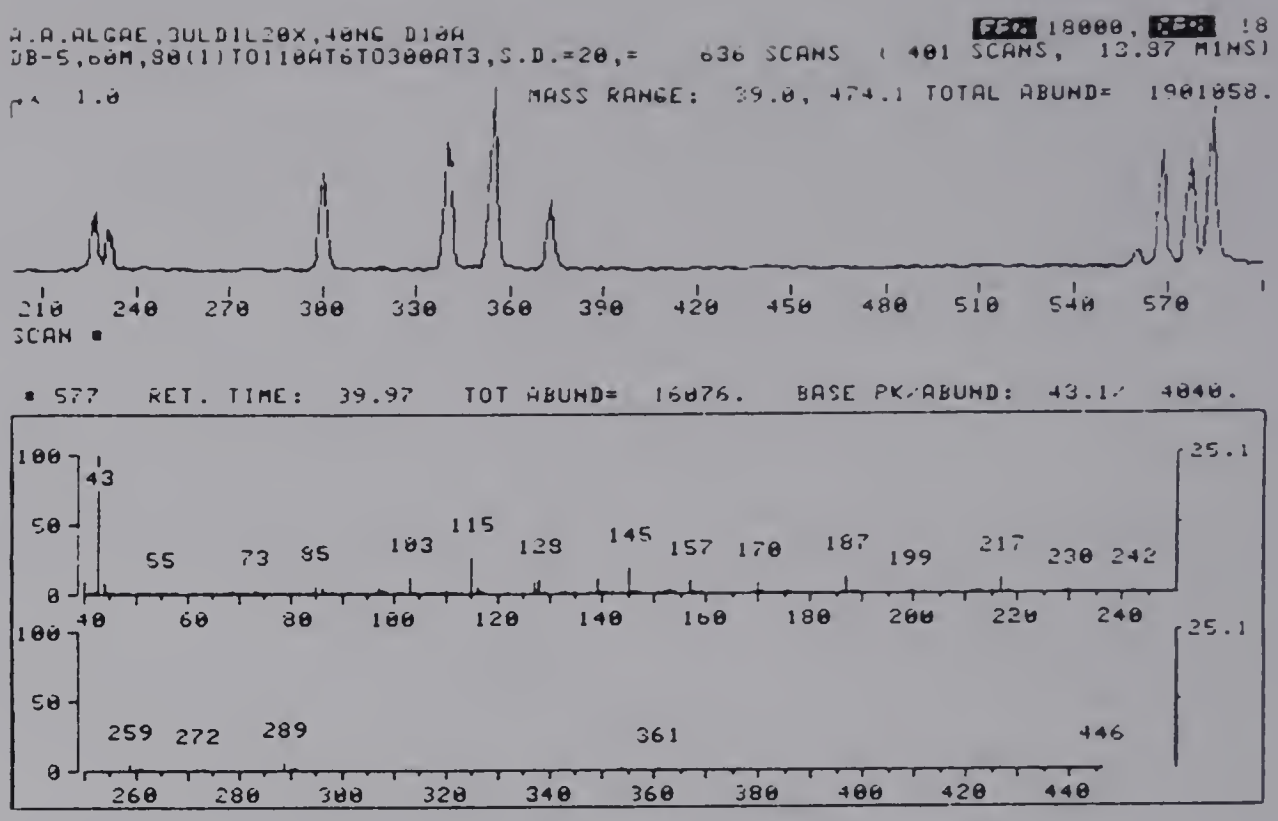


Figure 14G & H

Figure 14I & J

Electron impact mass spectra of (I) galactose and (J) glucose from the alditol acetate derivatization of FDS-W-no NO_3, hydrolyzed for 1 hour at 100 C in 2 N TFA.

I



J

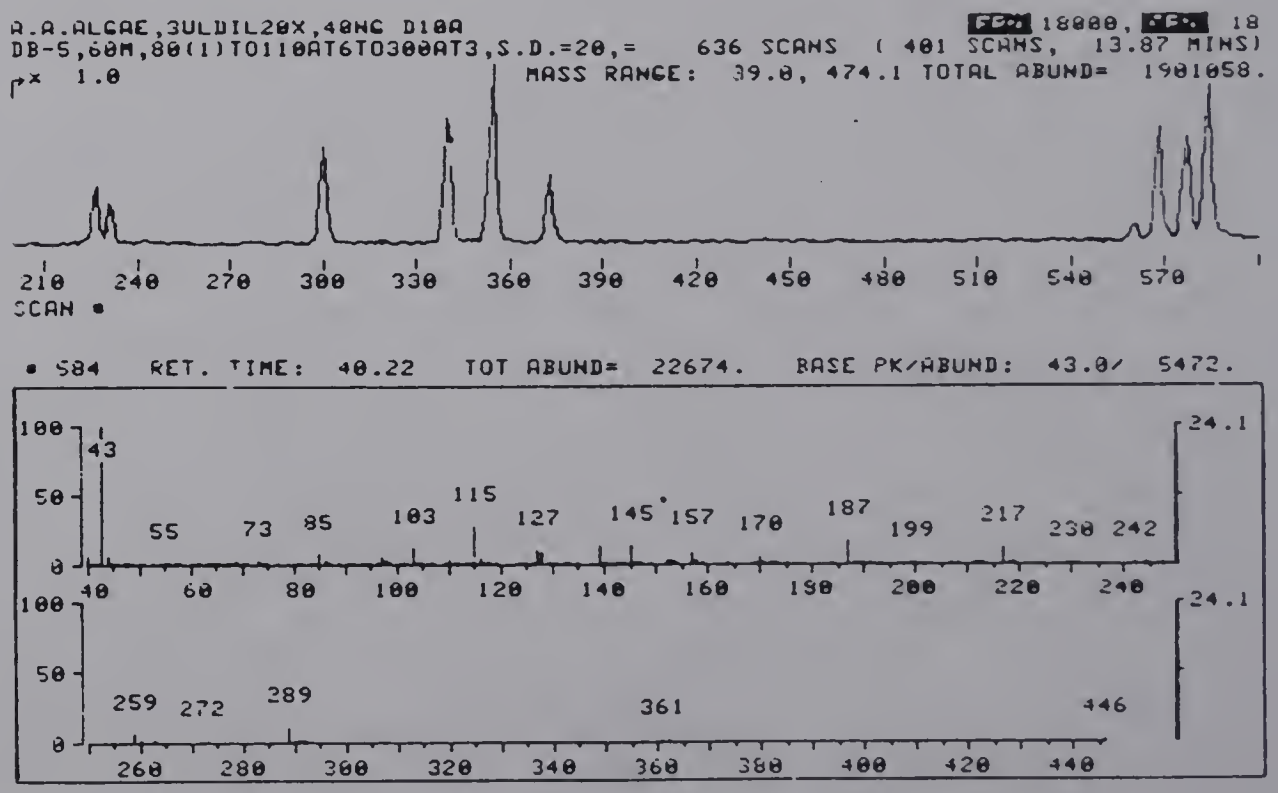


Figure 14I & J

Figure 14K

Electron impact mass spectra of a baseline blank.

K

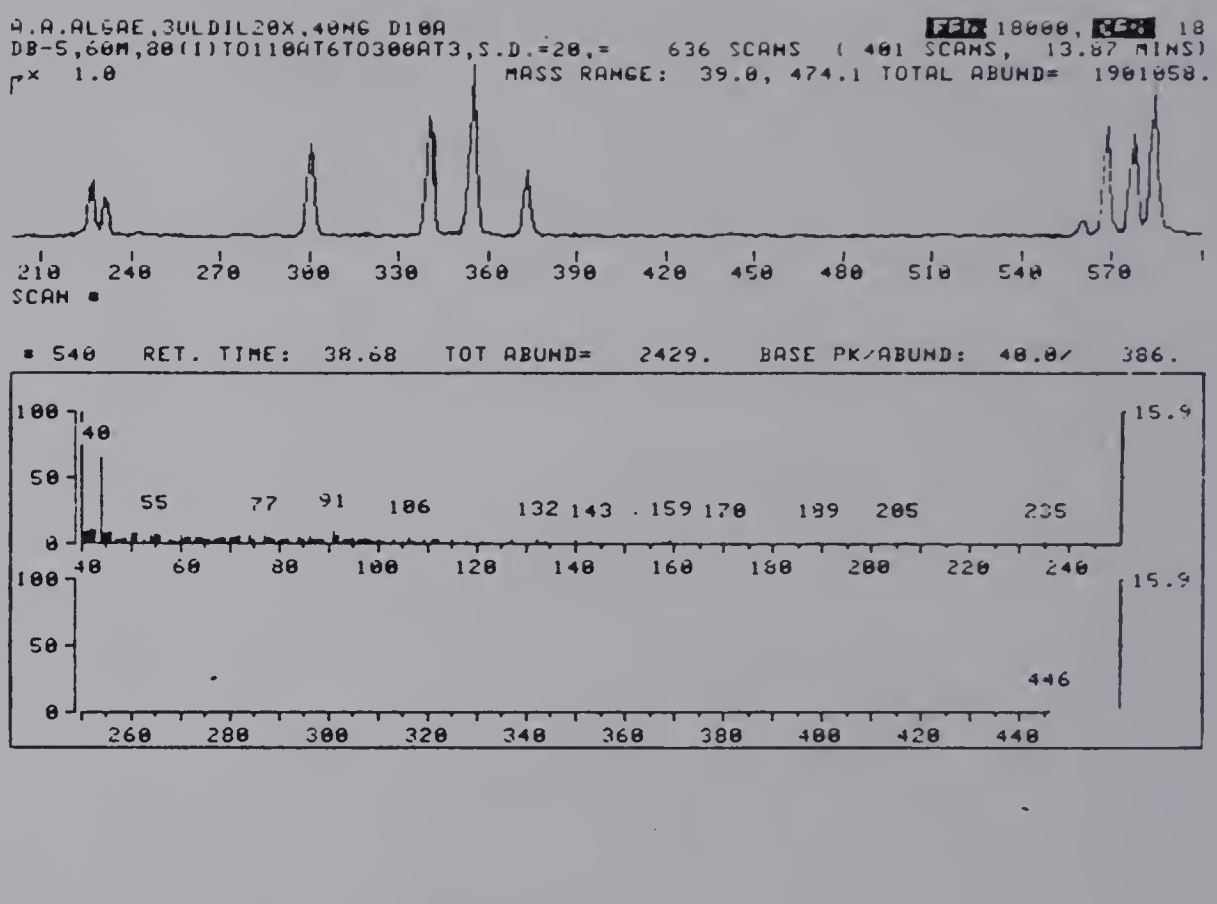
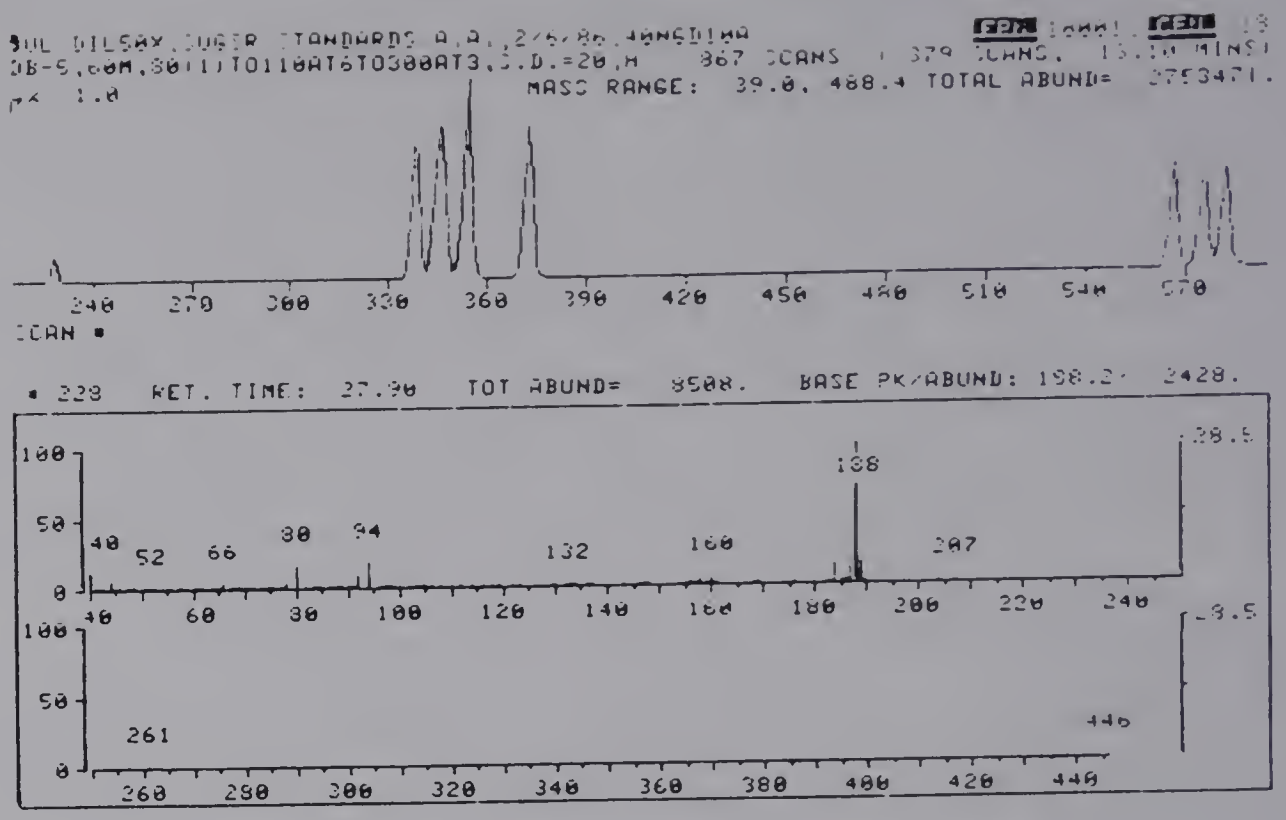

A.A.ALGAE,3ULDIL20X,40MG D10A
DB-5,60M,80(1)TO110AT6TO300AT3,S.D.=20,= 636 SCANS (401 SCANS, 13.87 MINS)
MASS RANGE: 39.0, 474.1 TOTAL ABUND= 1901058.
SCAN
• 540 RET. TIME: 38.68 TOT ABUND= 2429. BASE PK/ABUND: 40.0/ 386.
15.9
15.9
446

Figure 14K

Figure 15A & B

Electron impact mass spectra of (A) the internal GC-MS standard dioxane and (B) the alditol acetate derivative of the neutral sugar rhamnose.

A



B

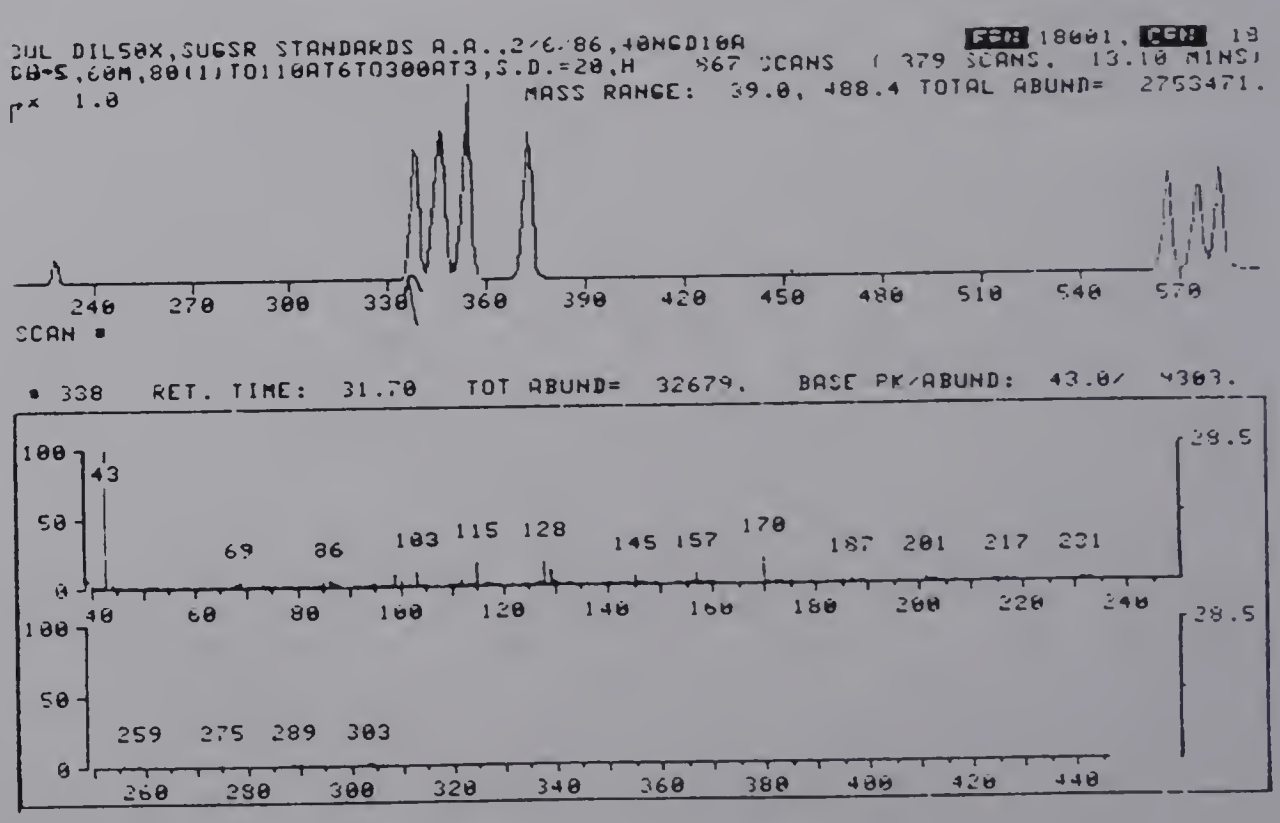
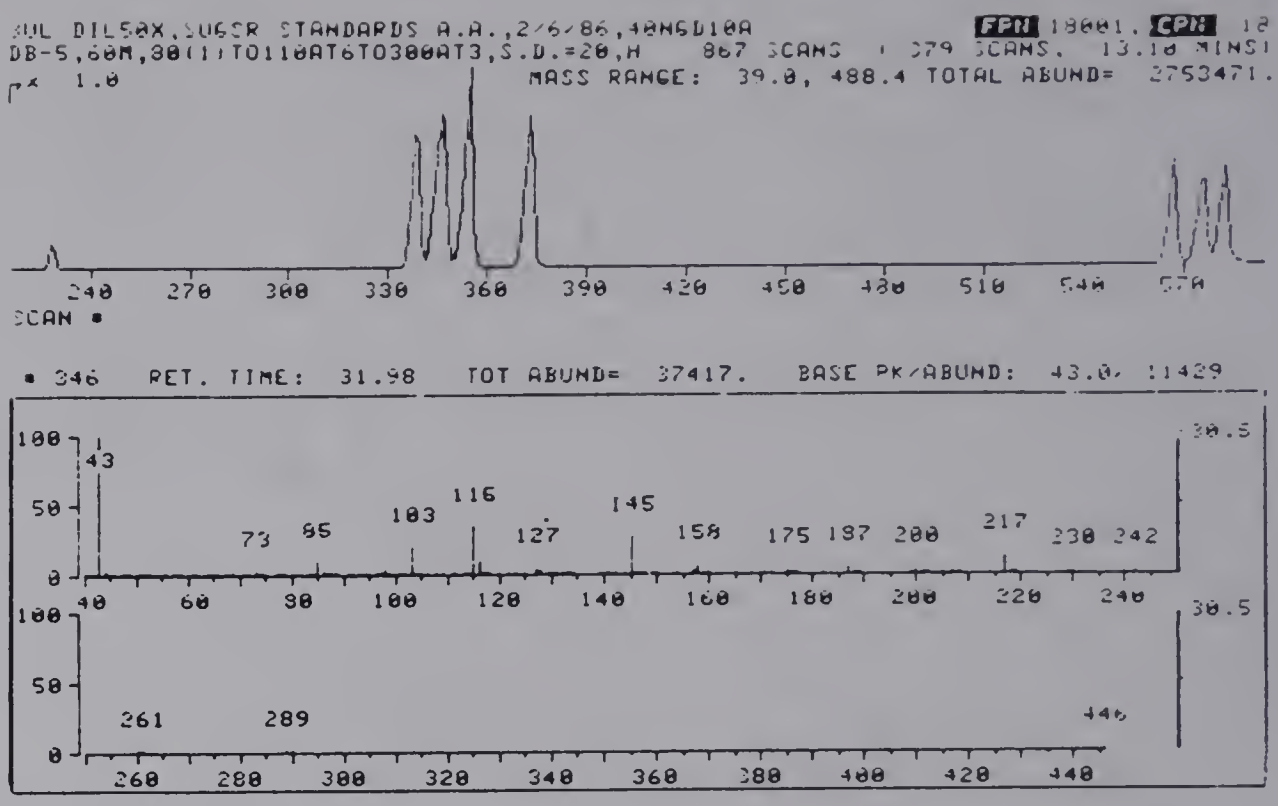


Figure 15A & B

Figure 15C & D

Electron impact mass spectra of the alditol acetate derivatives of the neutral sugars (C) ribose and (D) arabinose.

C



D

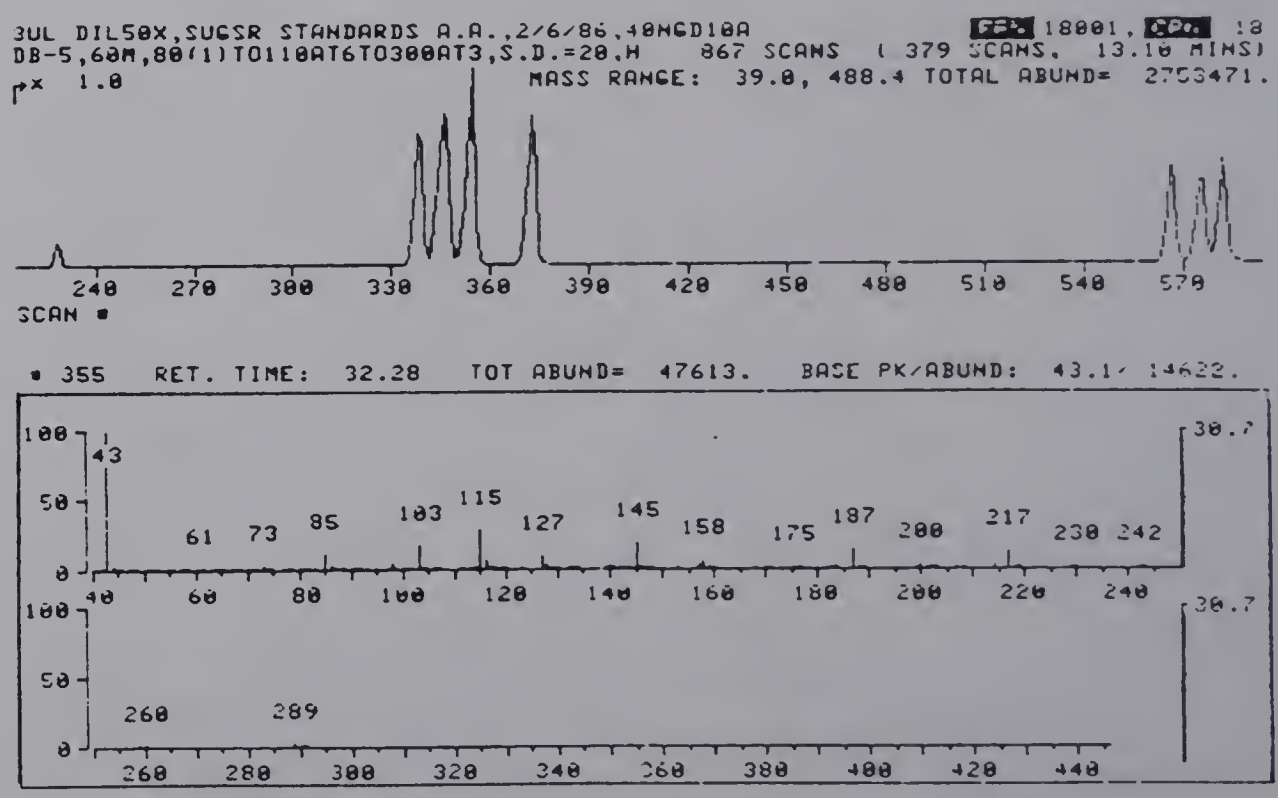
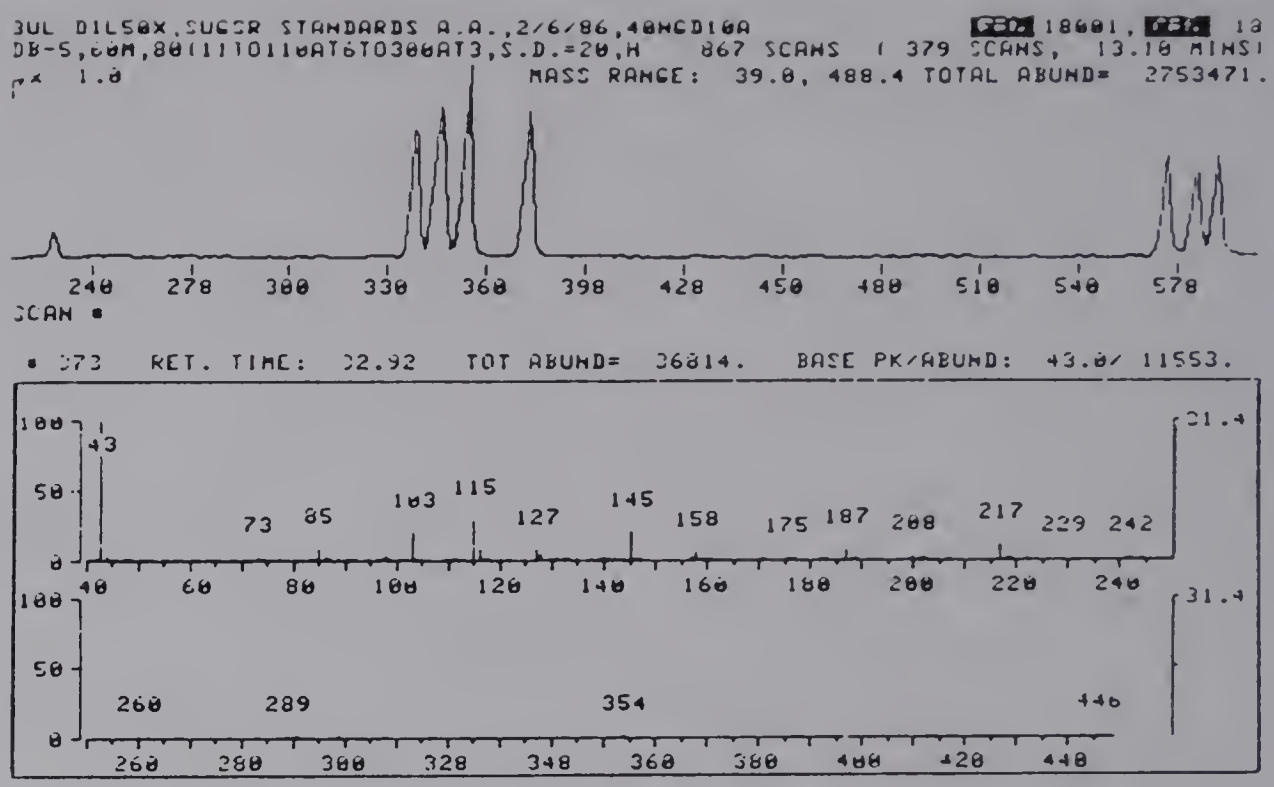


Figure 15C & D

Figure 15E & F

Electron impact mass spectra of the alditol acetate derivatives of the neutral sugars (E) xylose and (F) mannose.

E



F

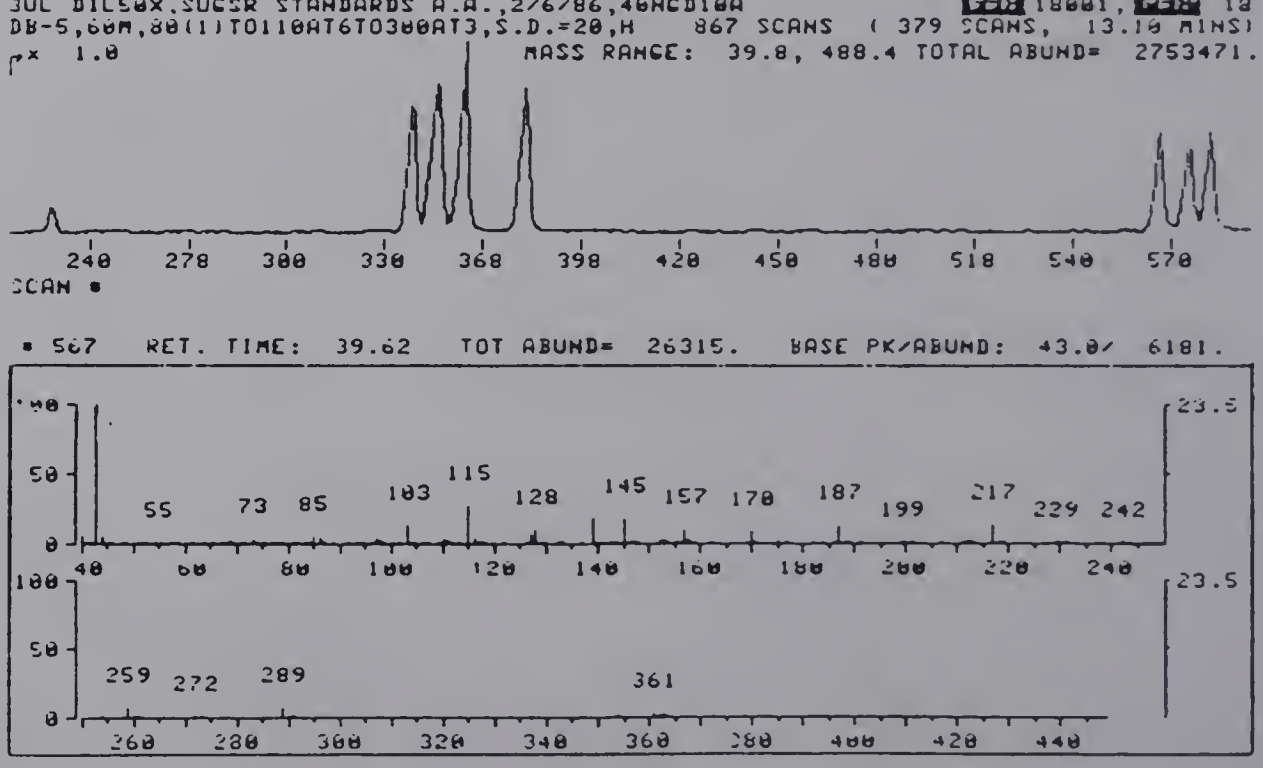
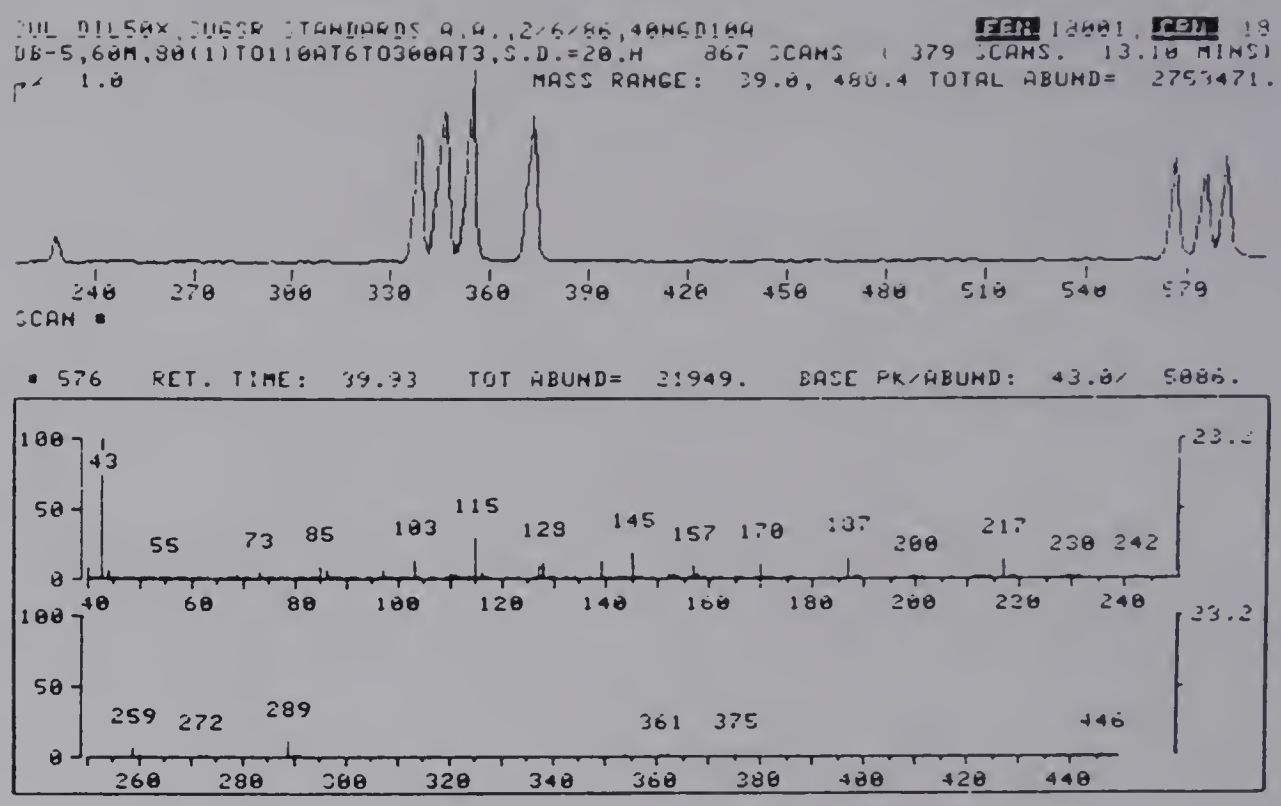


Figure 15E & F

Figure 15G & H

Electron impact mass spectra of the alditol acetate derivatives of the neutral sugars (G) galactose and (H) glucose.

G



H

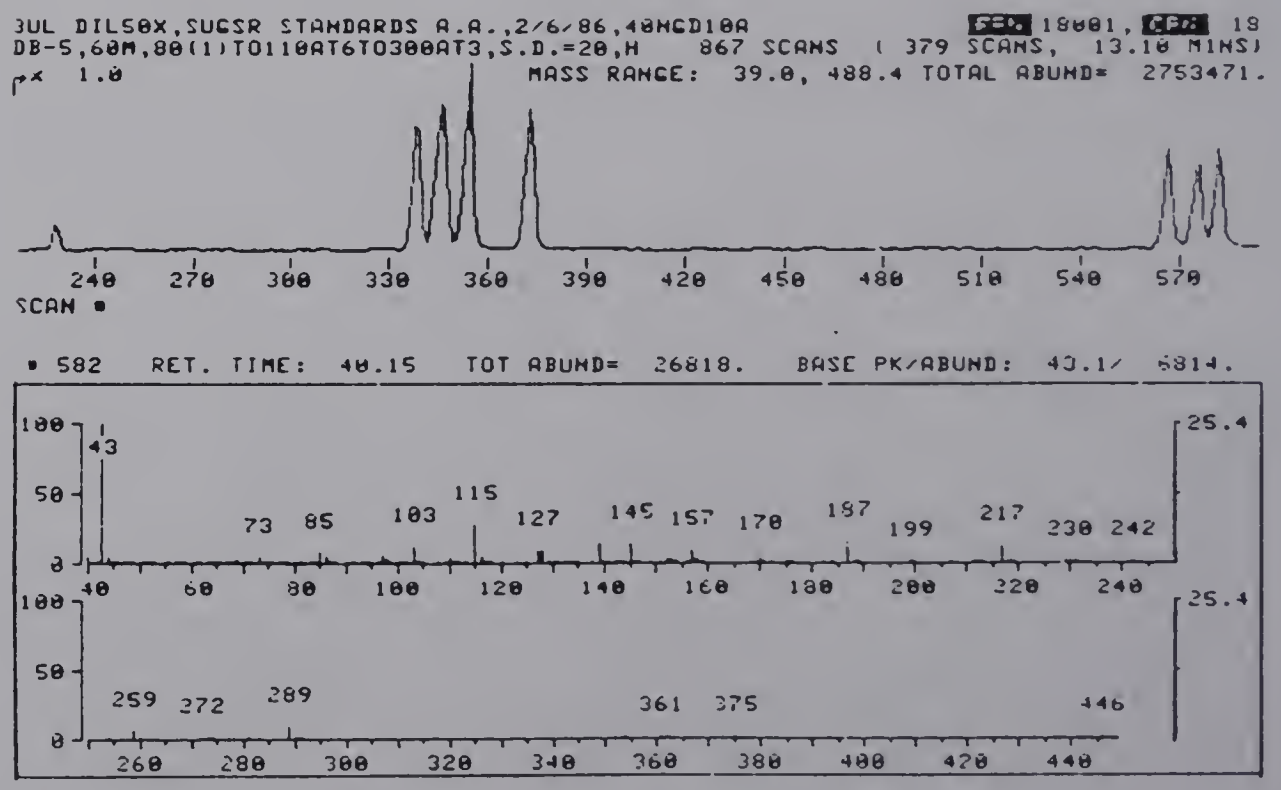


Figure 15G & H

Figure 15I

Electron impact mass spectra of a baseline blank.

I

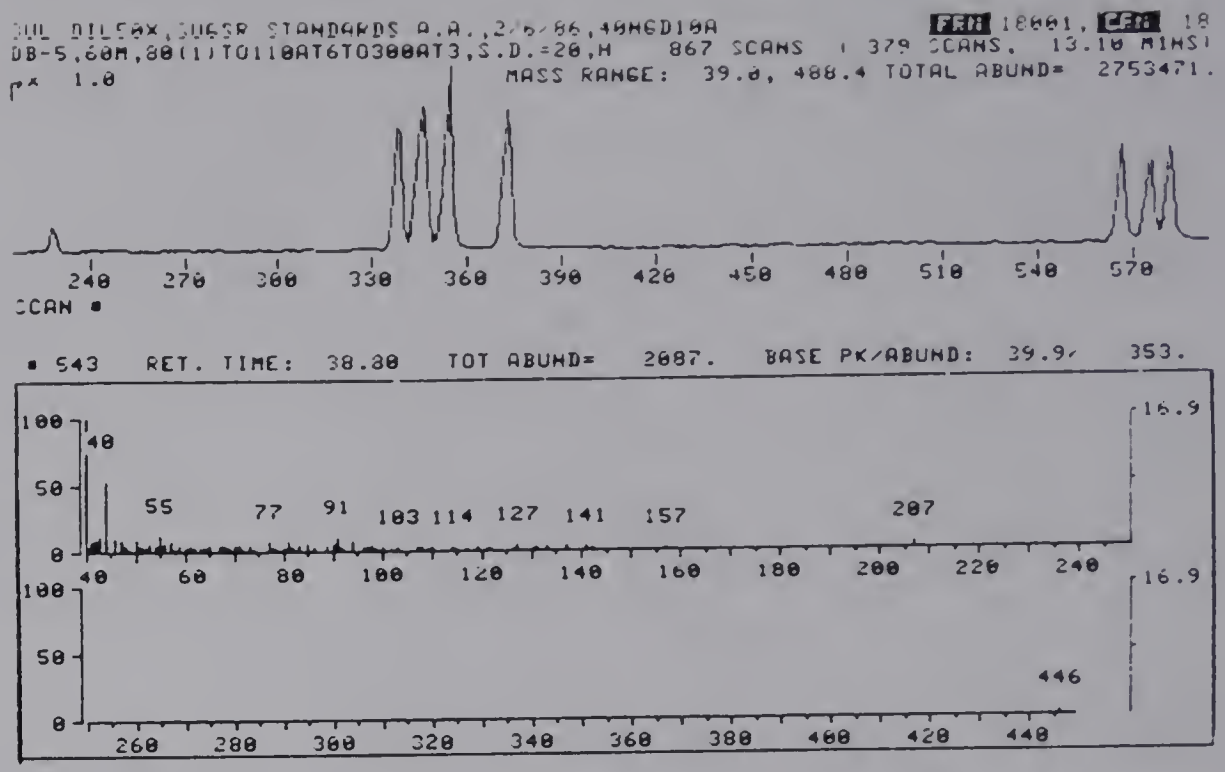
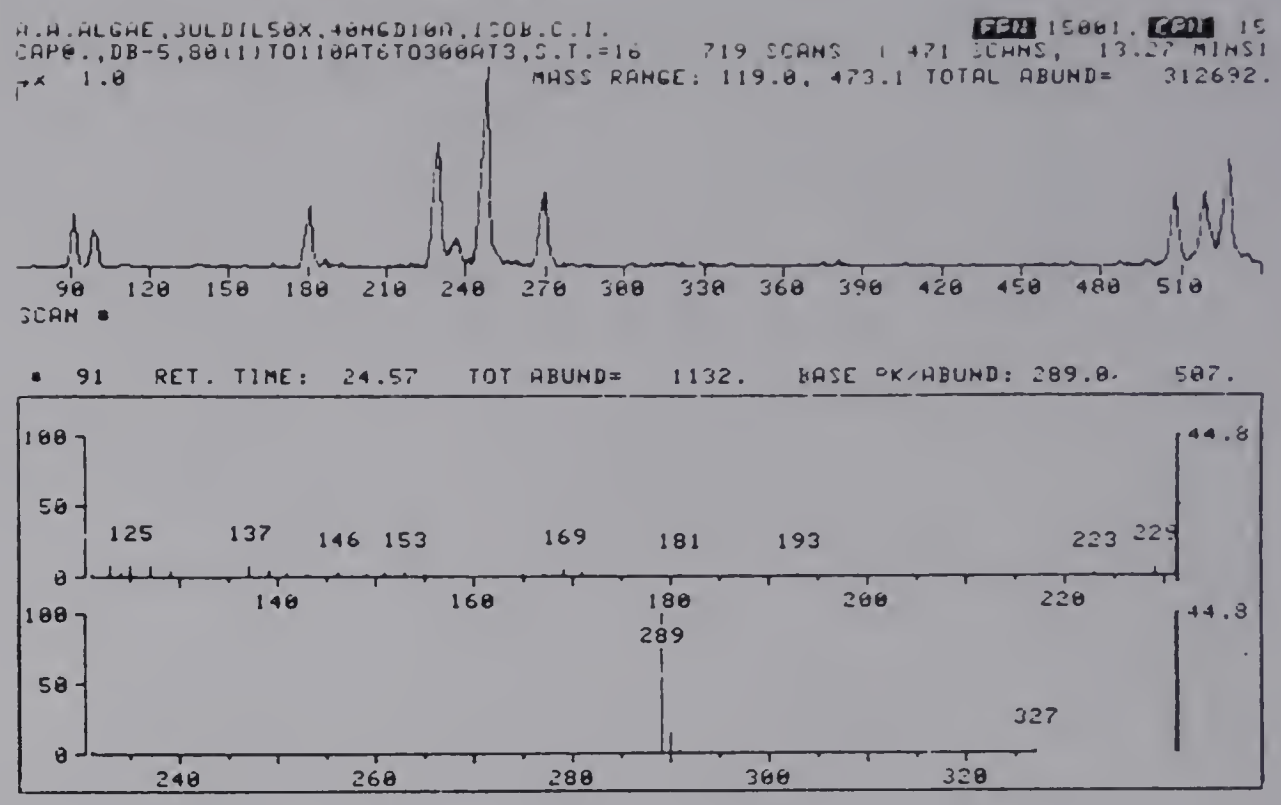

DB-5,60M,80(1)TO110AT6TO300AT3,S.D.=20,H
867 SCANS
MASS RANGE: 39.0, 488.4 TOTAL ABUND= 2753471.
SCAN #
543 RET. TIME: 38.80 TOT ABUND= 2087. BASE PK/ABUND: 39.9/ 353.
16.9
16.9

Figure 15I

Figure 16A & B

Isobutane chemical ionization mass spectra of (A) an unknown component and (B) the internal GC-MS standard dioxane from the alditol acetate derivatization of FDS-W-no NO_3 hydrolyzed in 2 N TFA for 1 hr at 100 C.

A



B

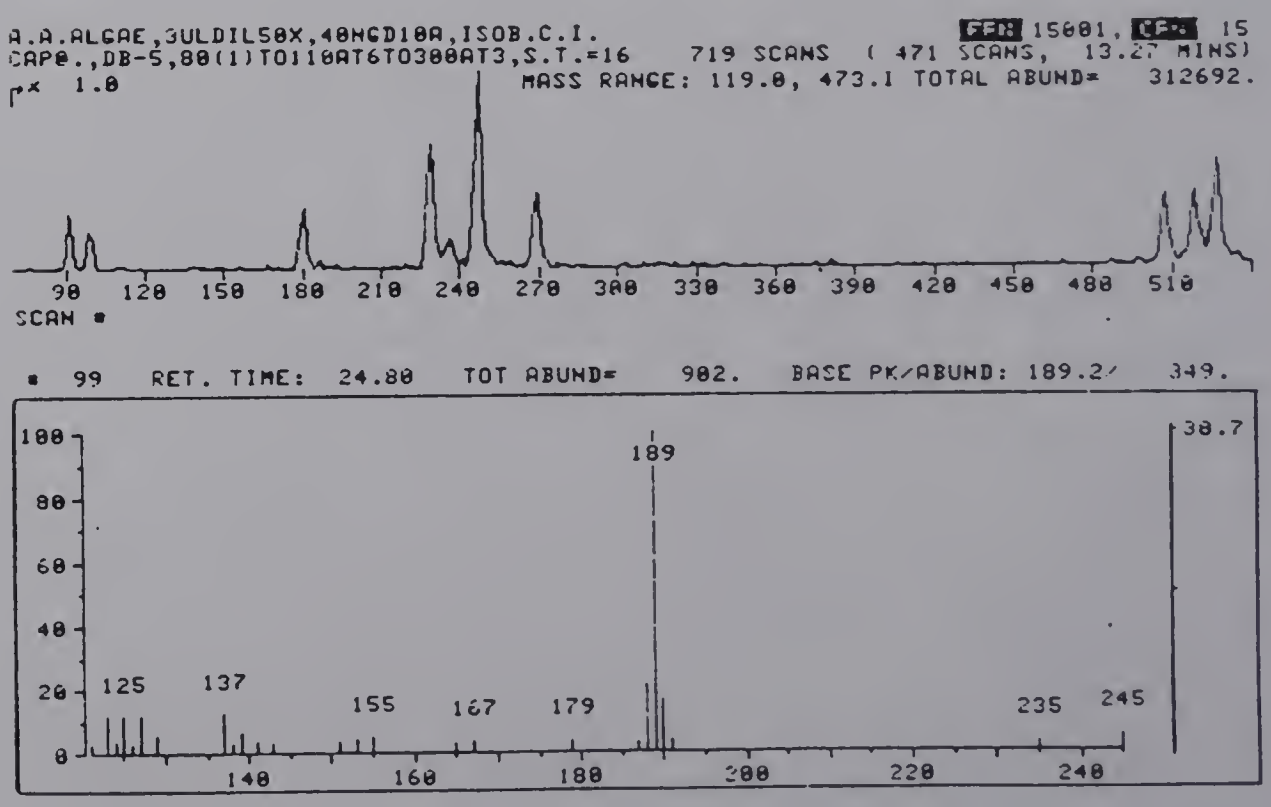
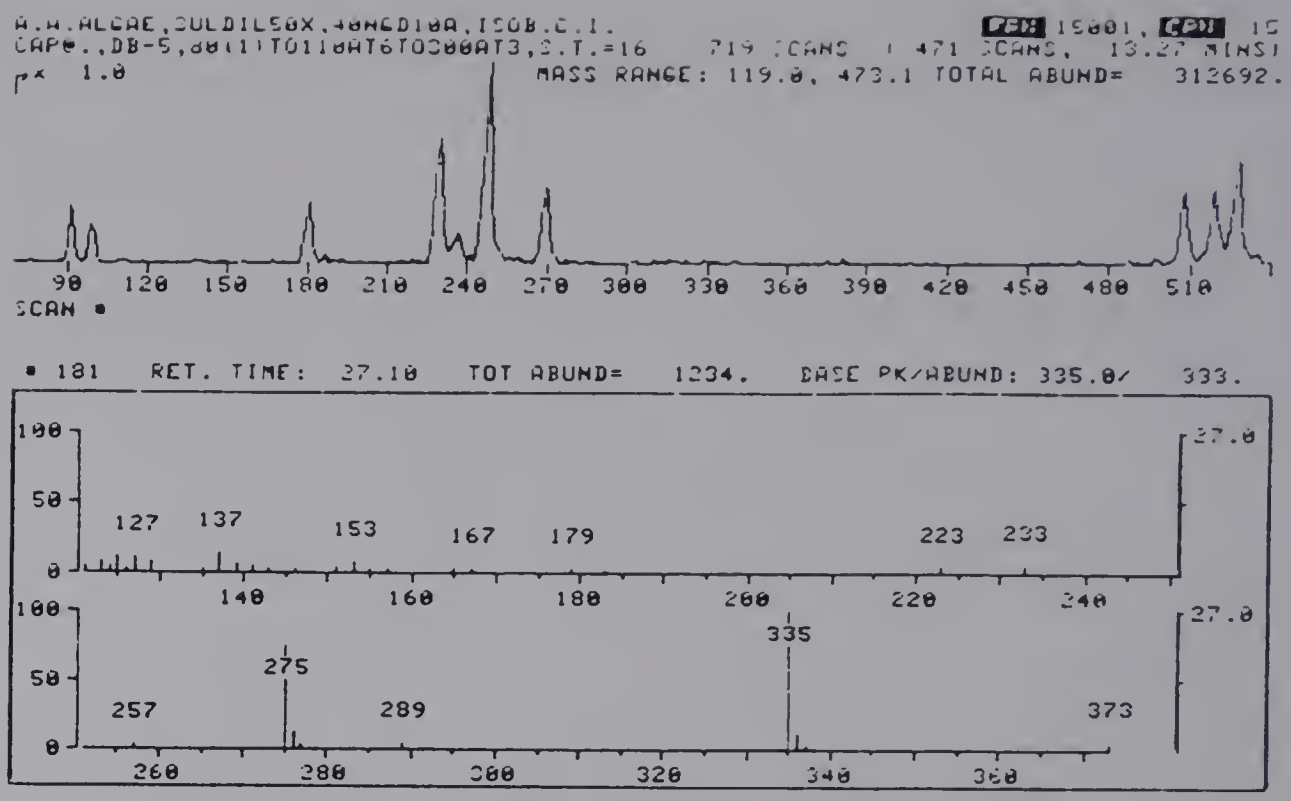


Figure 16A & B

Figure 16C & D

Isobutane chemical ionization mass spectra of (C) 2-O-methyl xylose (Mwt=334) and (D) rhamnose (Mwt=376) from the alditol acetate derivatization of FDS-W-no NO_3 hydrolyzed in 2 N TFA for 1 hr at 100 C.

C



D

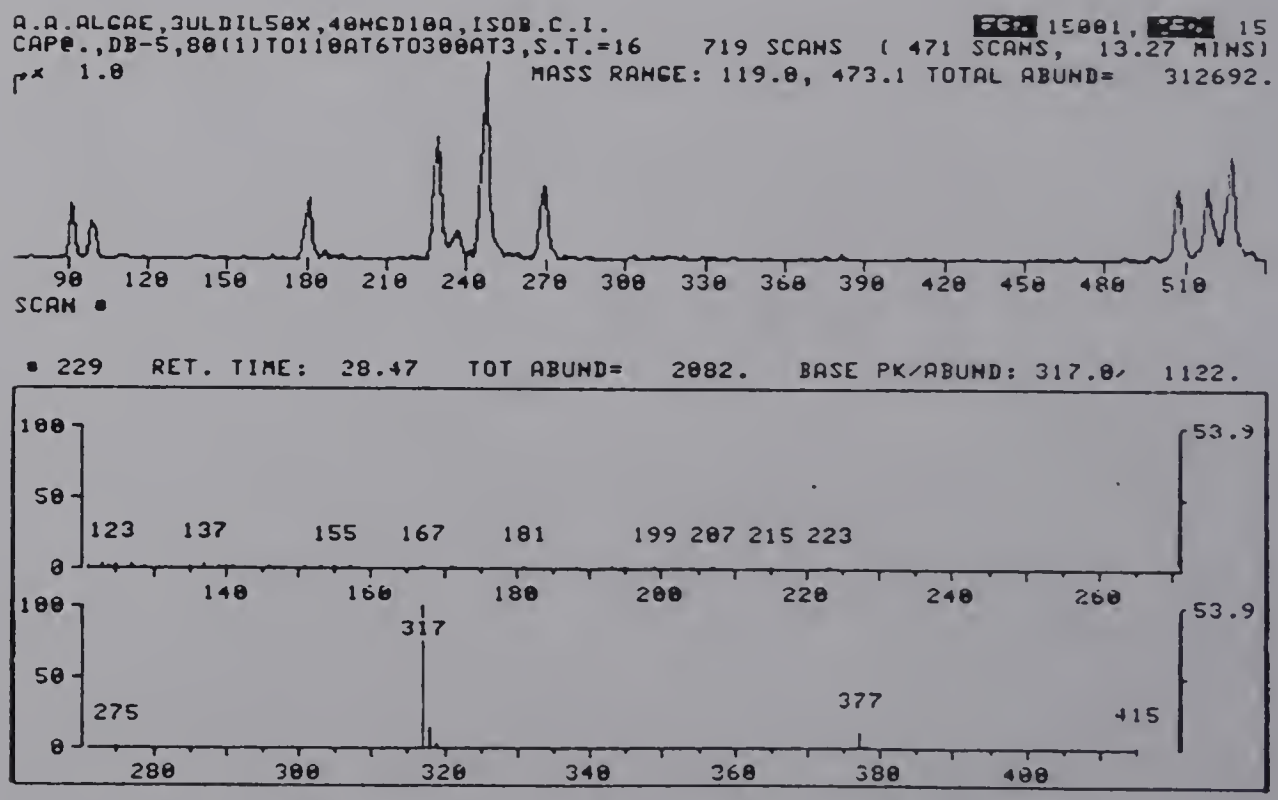
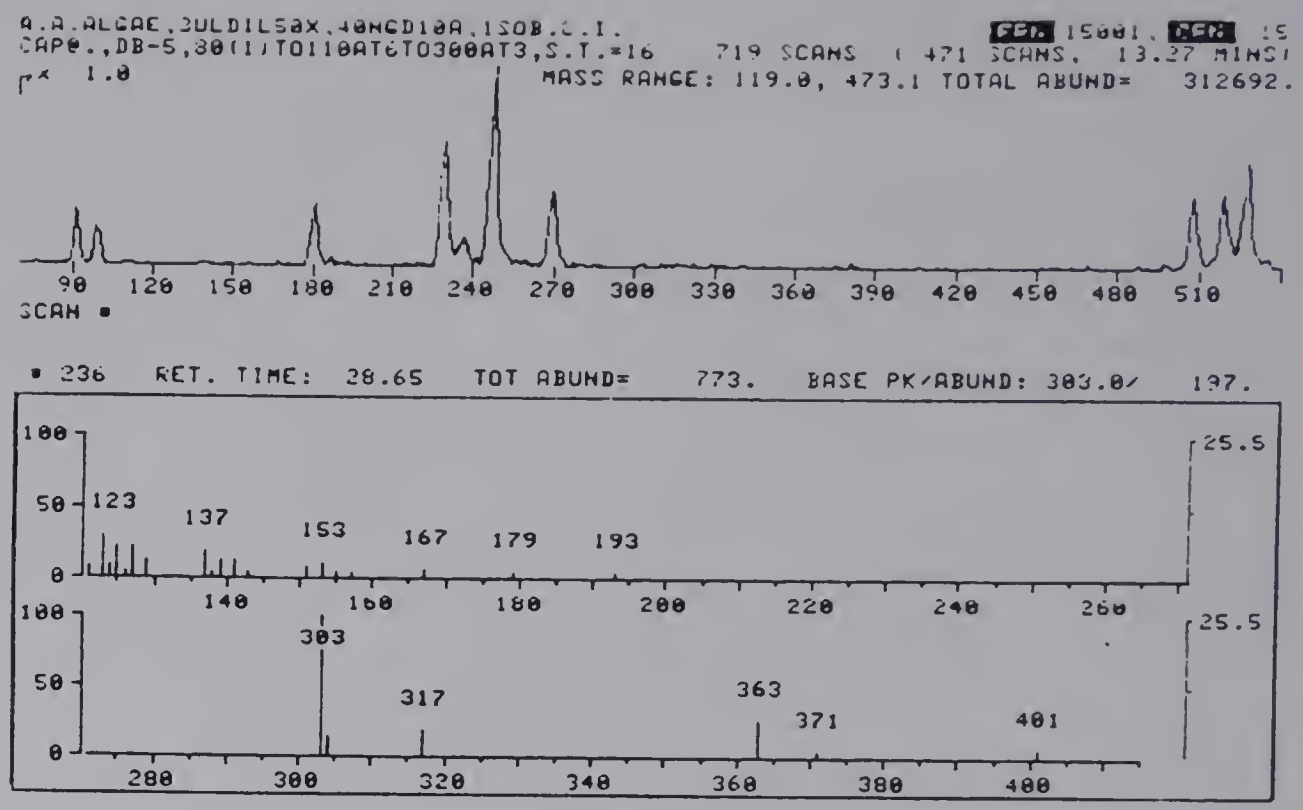


Figure 16C & D

Figure 16E & F

Isobutane chemical ionization mass spectra of (E) an unknown component (Mwt=362) and (F) arabinose (Mwt=362) (internal GC standard) from the alditol acetate derivatization of FDS-W-no NO_3 hydrolyzed in 2 N TFA for 1 hr at 100 C.

E



F

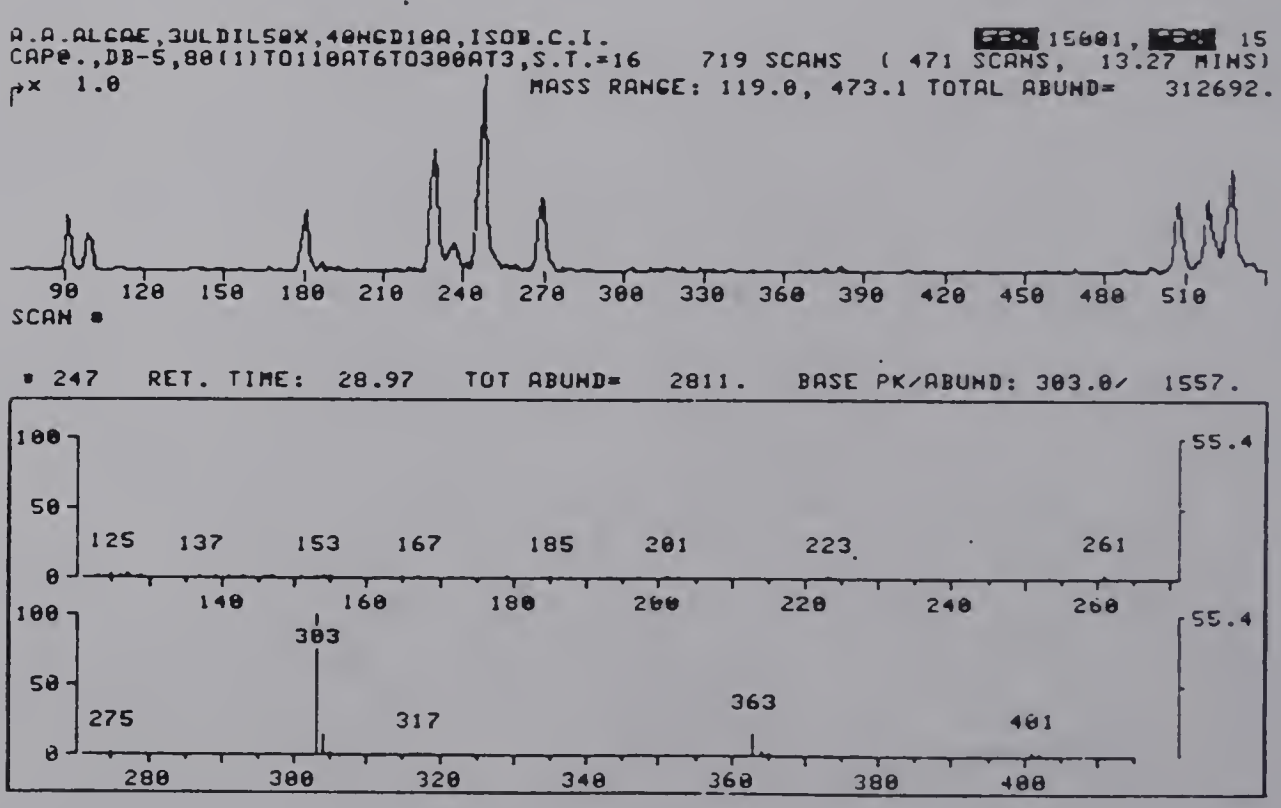
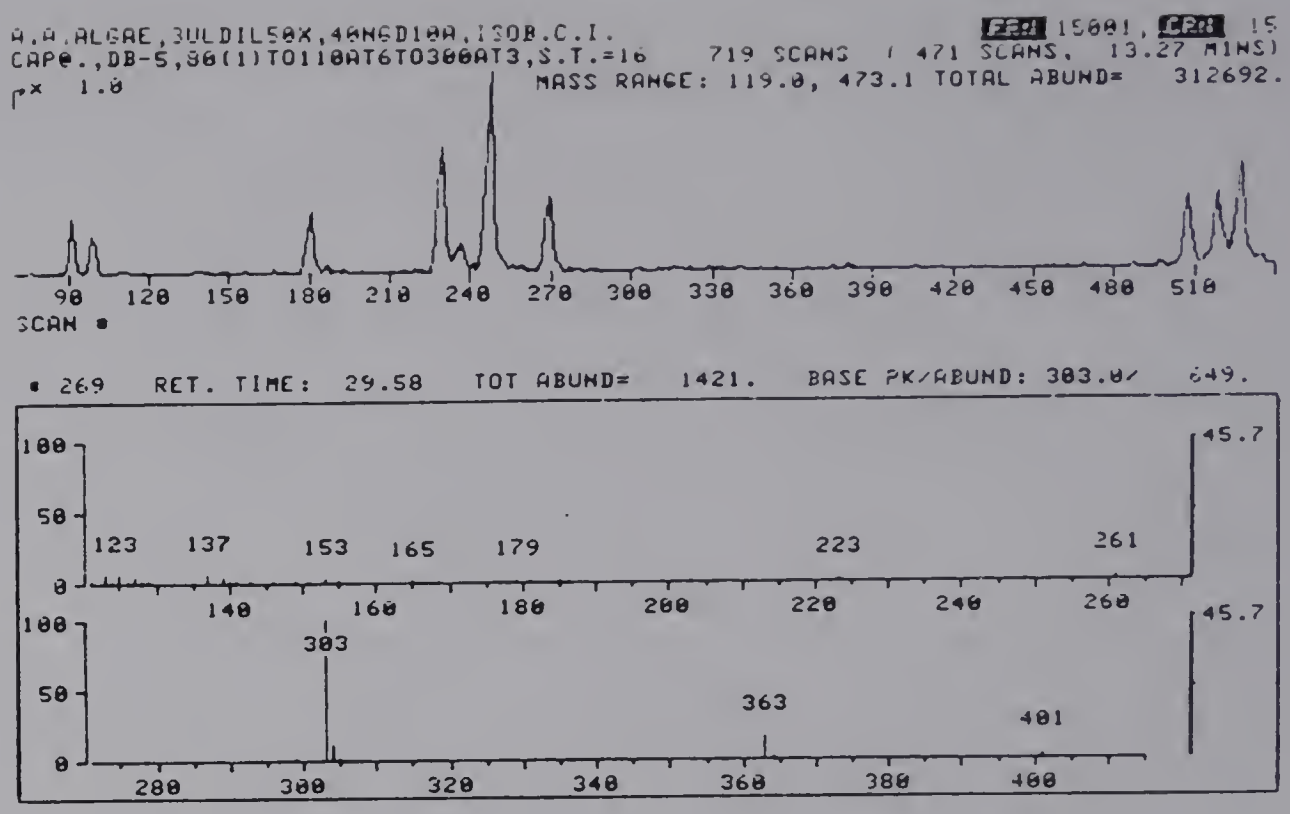


Figure 16E & F

Figure 16G & H

Isobutane chemical ionization mass spectra of (G) xylose (Mwt=362) and (H) mannose (Mwt=434) from the alditol acetate derivatization of FDS-W-no NO_3 hydrolyzed in 2 N TFA for 1 hr at 100 C.

G



H

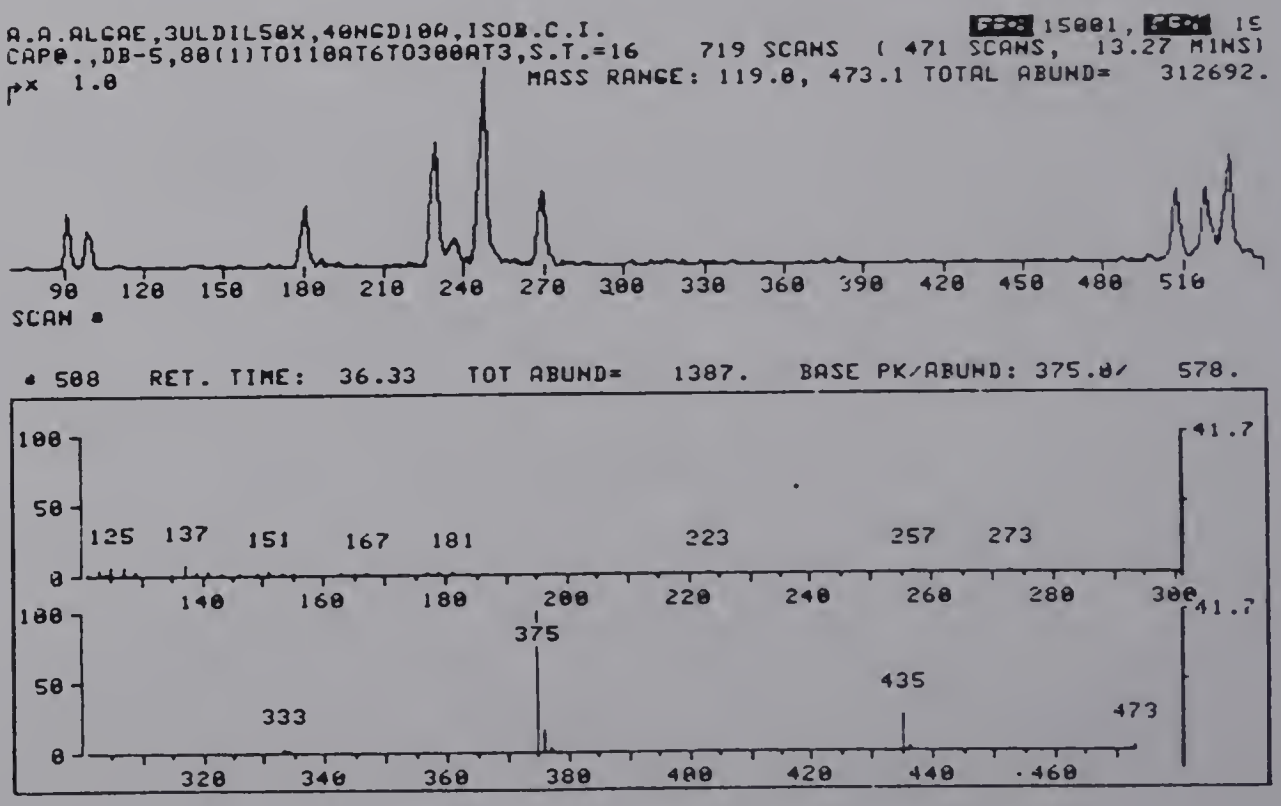
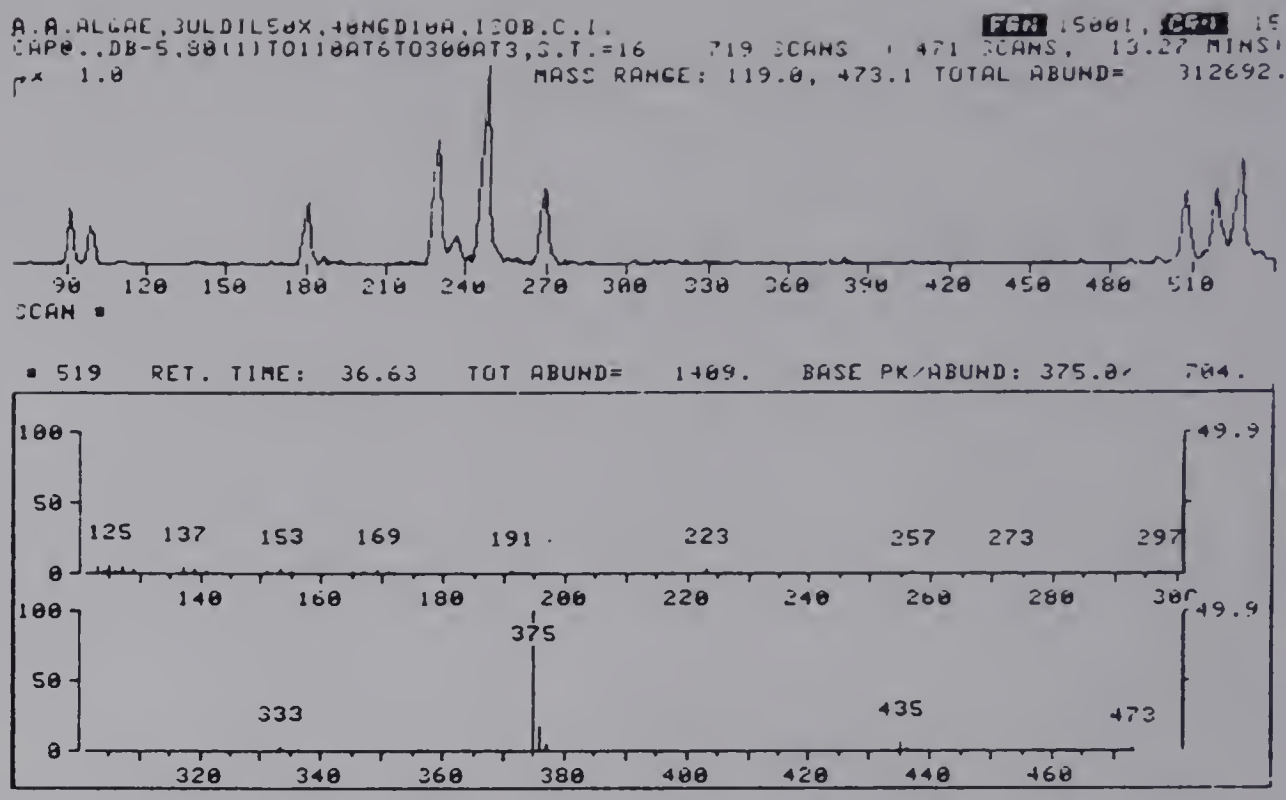


Figure 16G & H

Figure 16I & J

Isobutane chemical ionization mass spectra of (I) galactose (Mwt=434) and (J) glucose (Mwt=434) from the alditol acetate derivatization of FDS-W-no NO_3 hydrolyzed in 2 N TFA for 1 hr at 100 C.

I



J

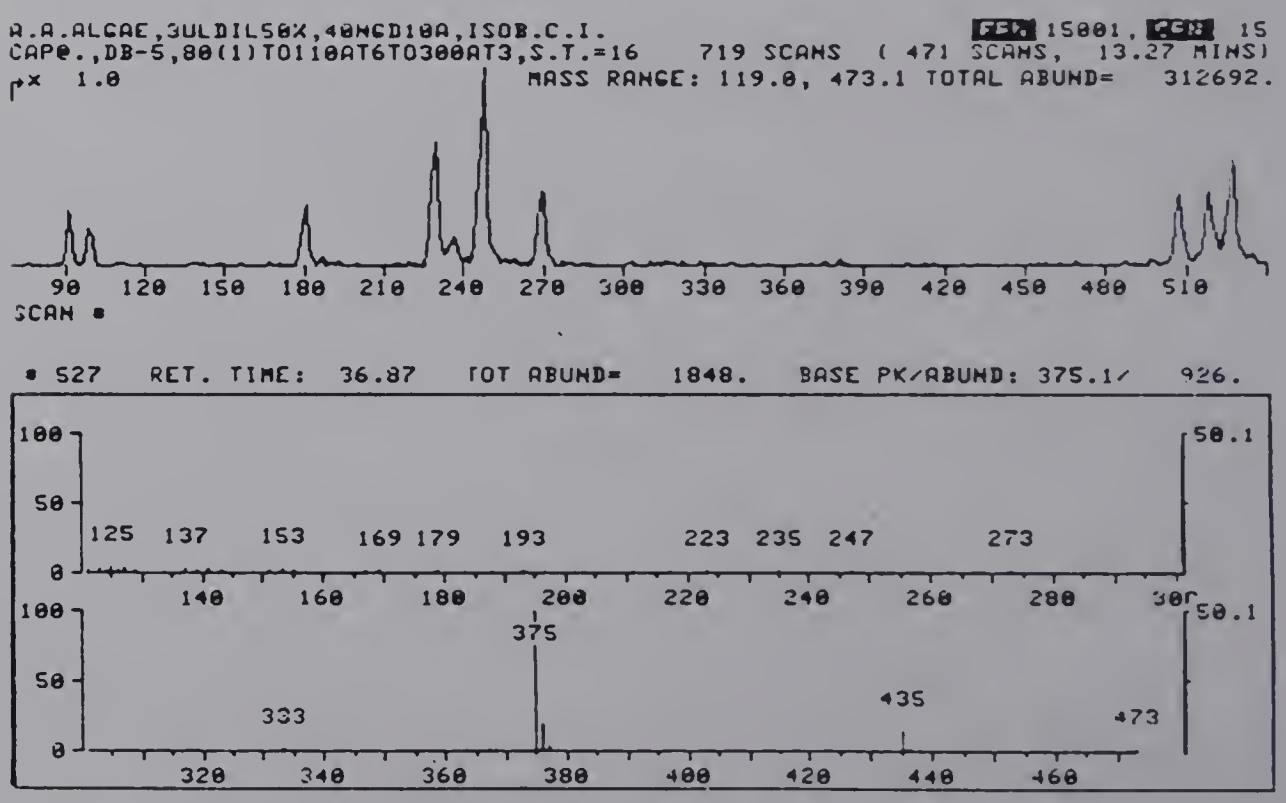
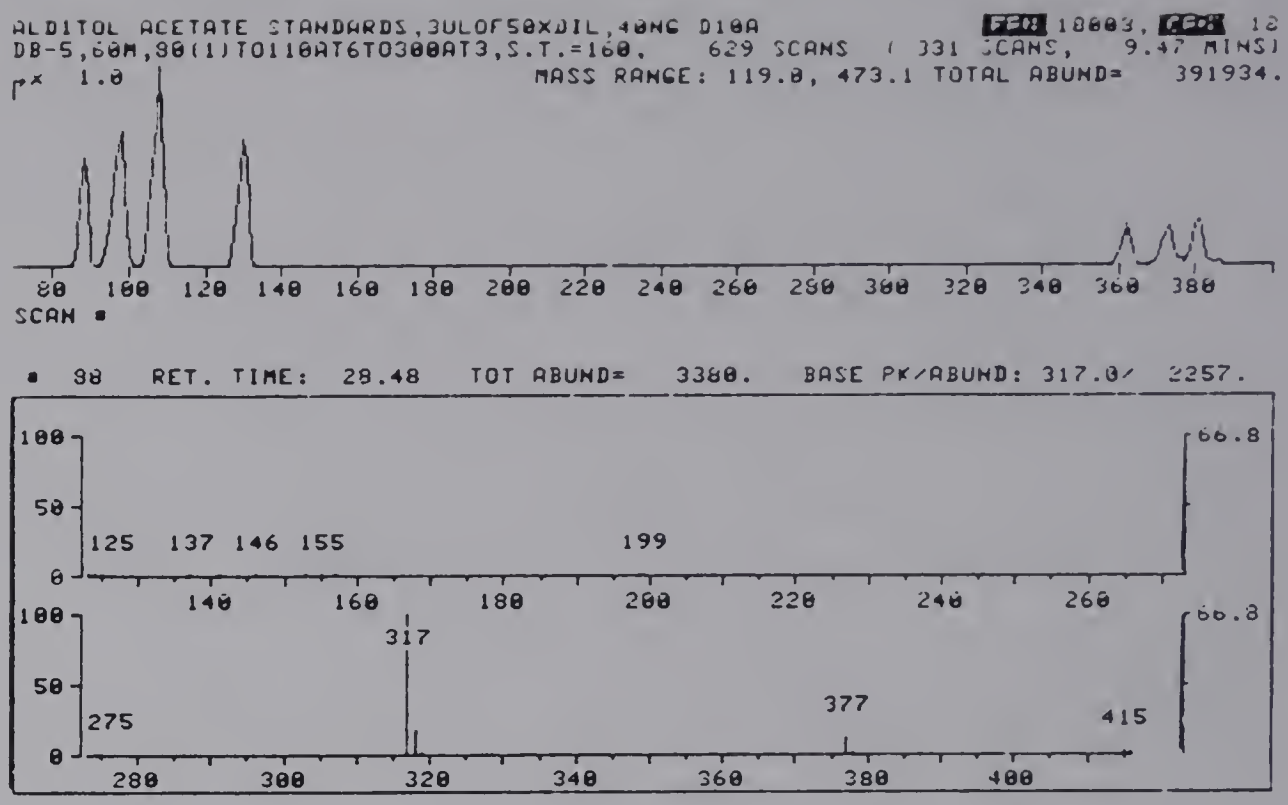


Figure 16I & J

Figure 17A & B

Isobutane chemical ionization mass spectra of the alditol acetate derivatives of the neutral sugars (A) rhamnose (Mwt=376) and (B) ribose (Mwt=362).

A



B

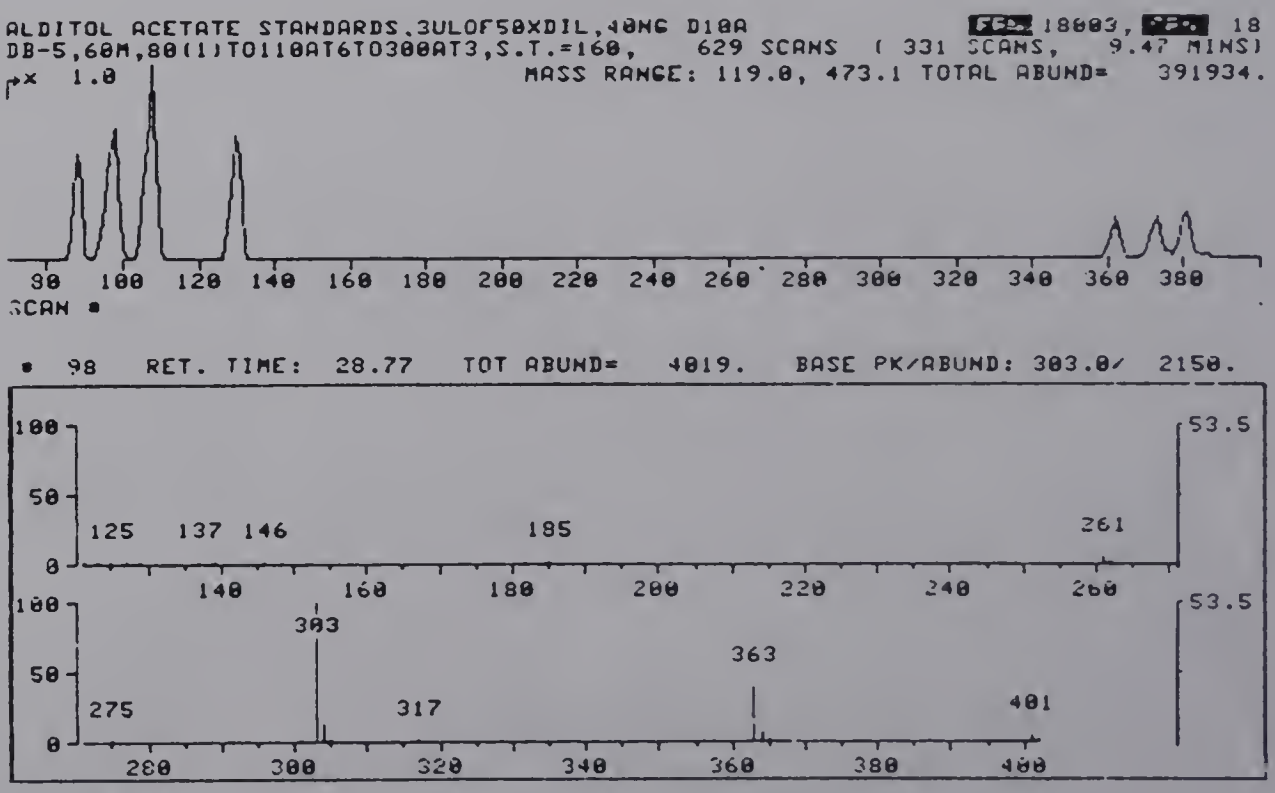


Figure 17A & B

Figure 17C

Isobutane chemical ionization mass spectra of the alditol acetate derivatives of the neutral sugar arabinose (Mwt=362).

C

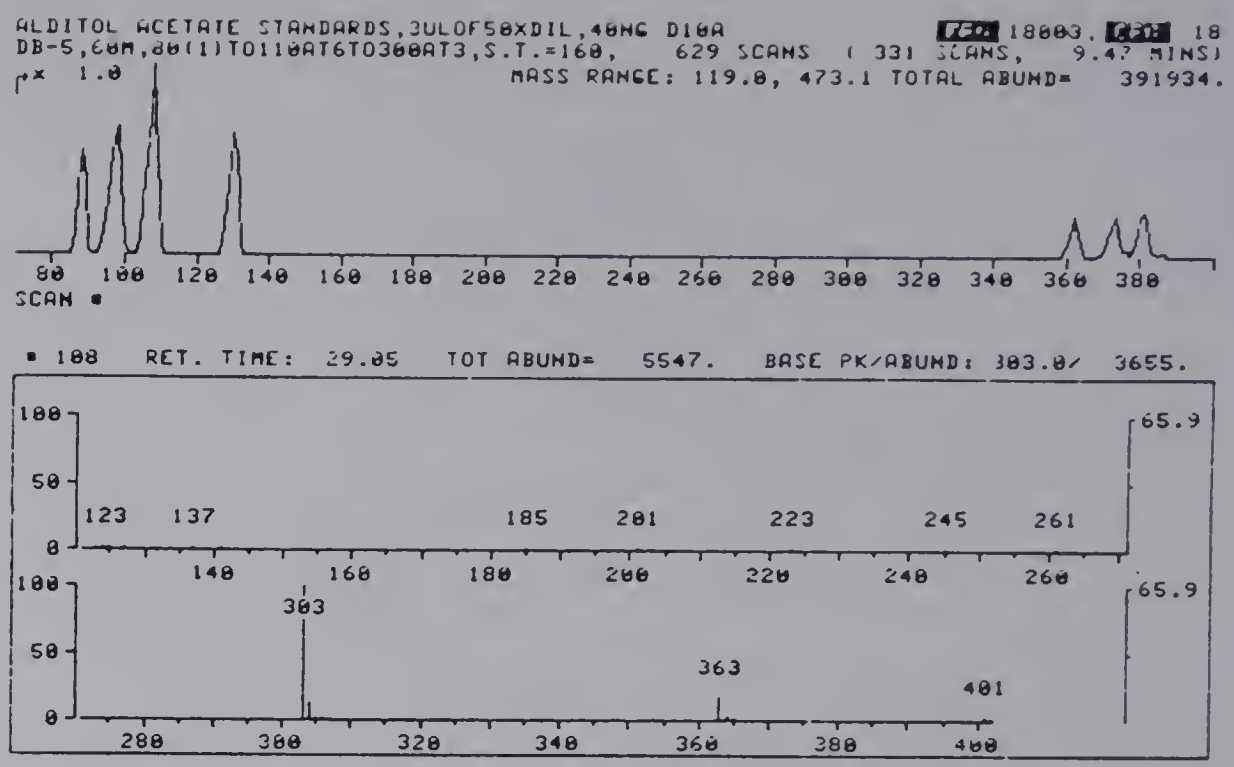
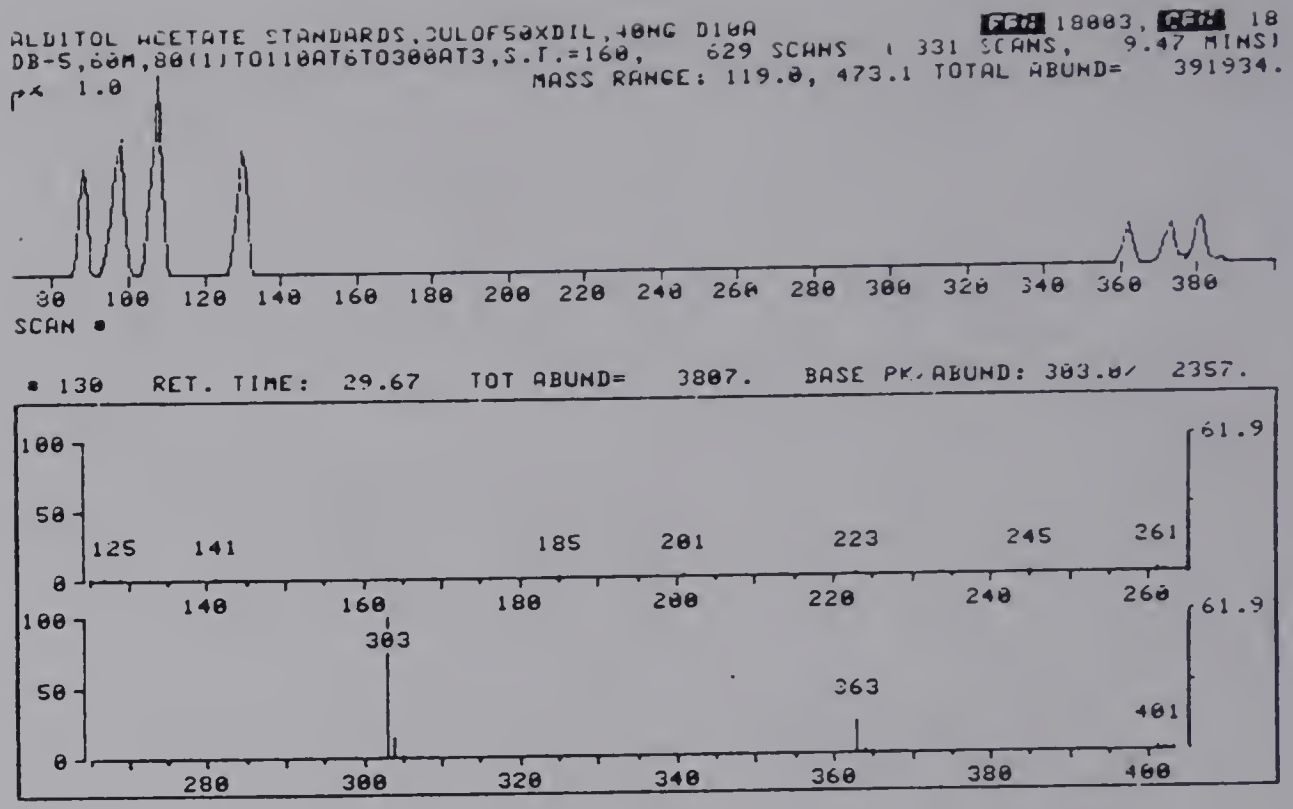

ALDITOL ACETATE STANDARDS,3ULOF50XDIL,40NG D10A
DB-5,60M,80(1)TO110AT6TO300AT3,S.T.=160,
629 SCANS (331 SCANS, 9.47 MINS)
MASS RANGE: 119.0, 473.1 TOTAL ABUND= 391934.
1.0
80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380
SCAN #
100 RET. TIME: 29.05 TOT ABUND= 5547. BASE PK/ABUND: 303.0/ 3655.
100
50
0
123 137 185 201 223 245 261
65.9
140 160 180 200 220 240 260
303
363
401
65.9
280 300 320 340 360 380 400

Figure 17C

Figure 17D & E

Isobutane chemical ionization mass spectra of the alditol acetate derivatives of the neutral sugars (D) xylose (Mwt=362) and (E) mannose (Mwt=434).

D



E

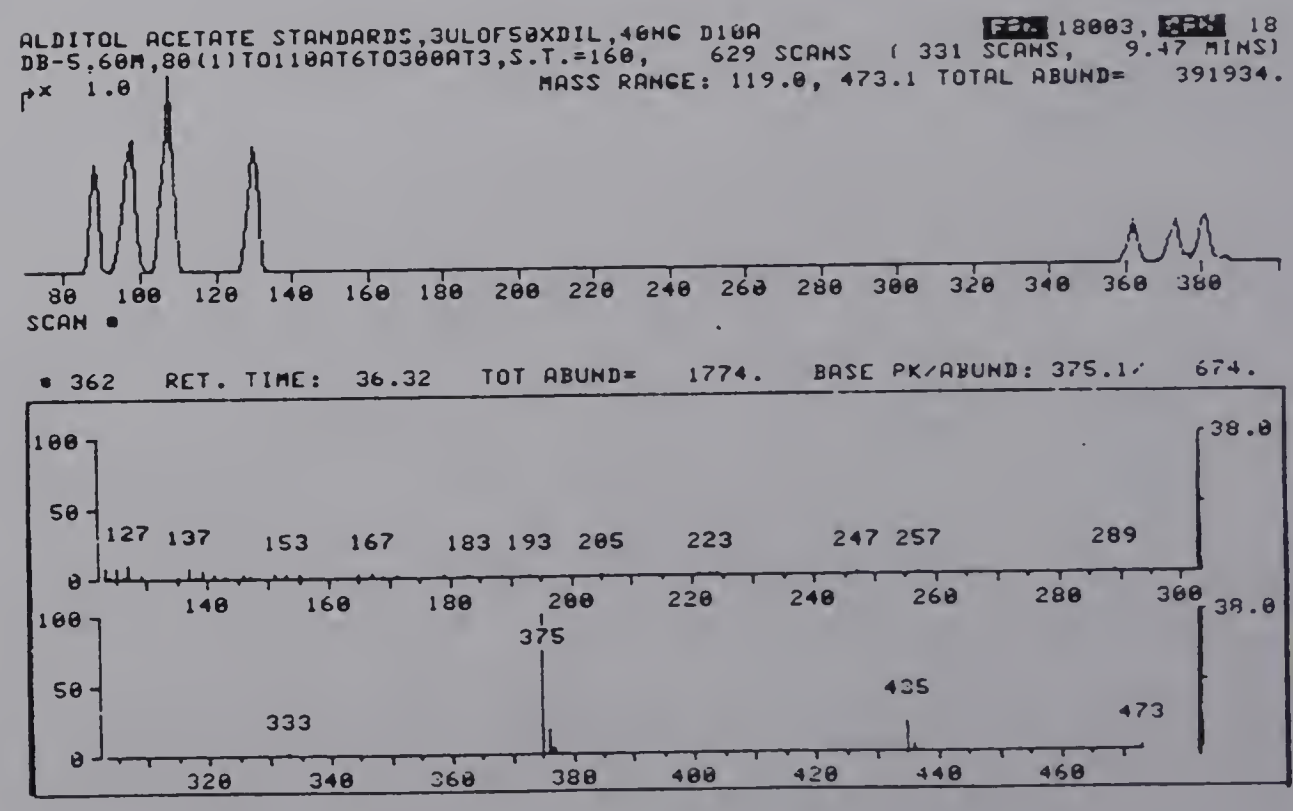
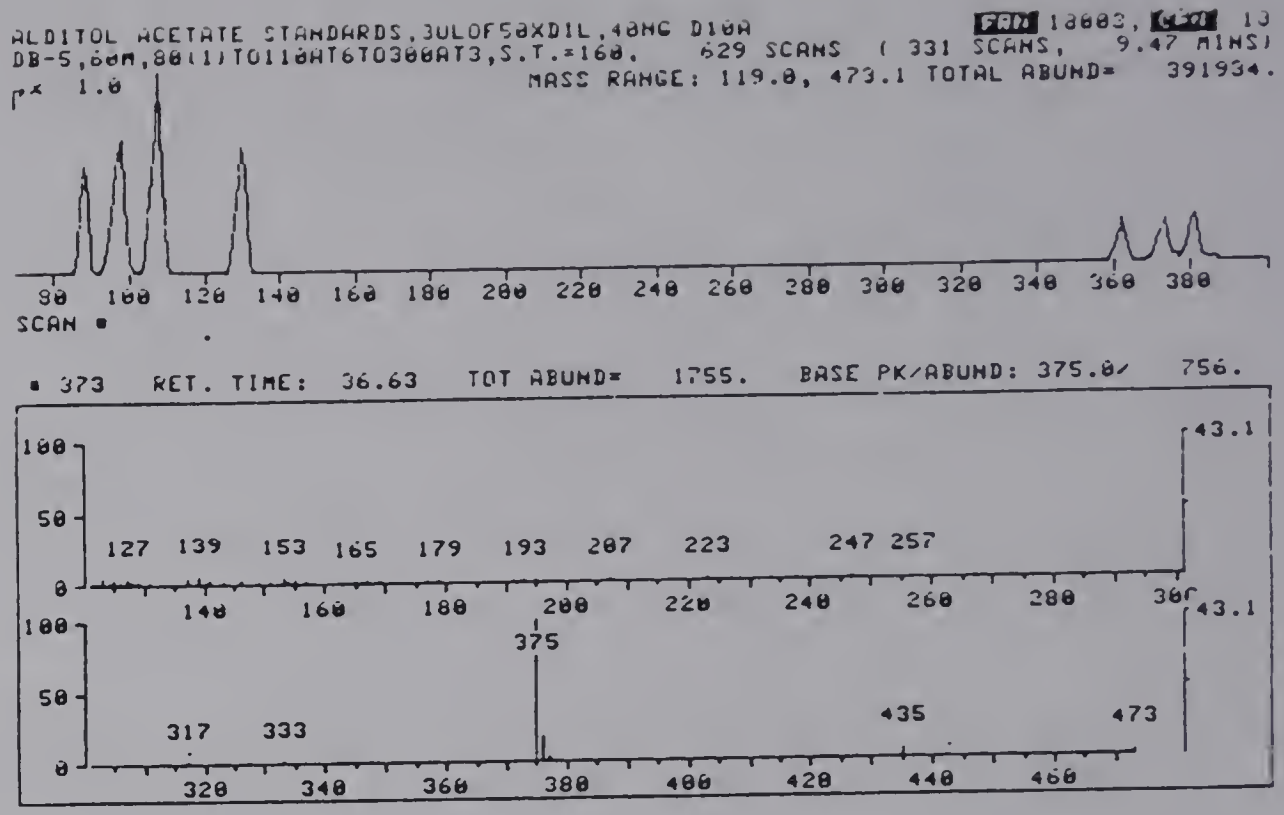


Figure 17D & E

Figure 17F & G

Isobutane chemical ionization mass spectra of the alditol acetate derivatives of the neutral sugars (F) galactose (Mwt=434) and (G) glucose (Mwt=434).

F



G

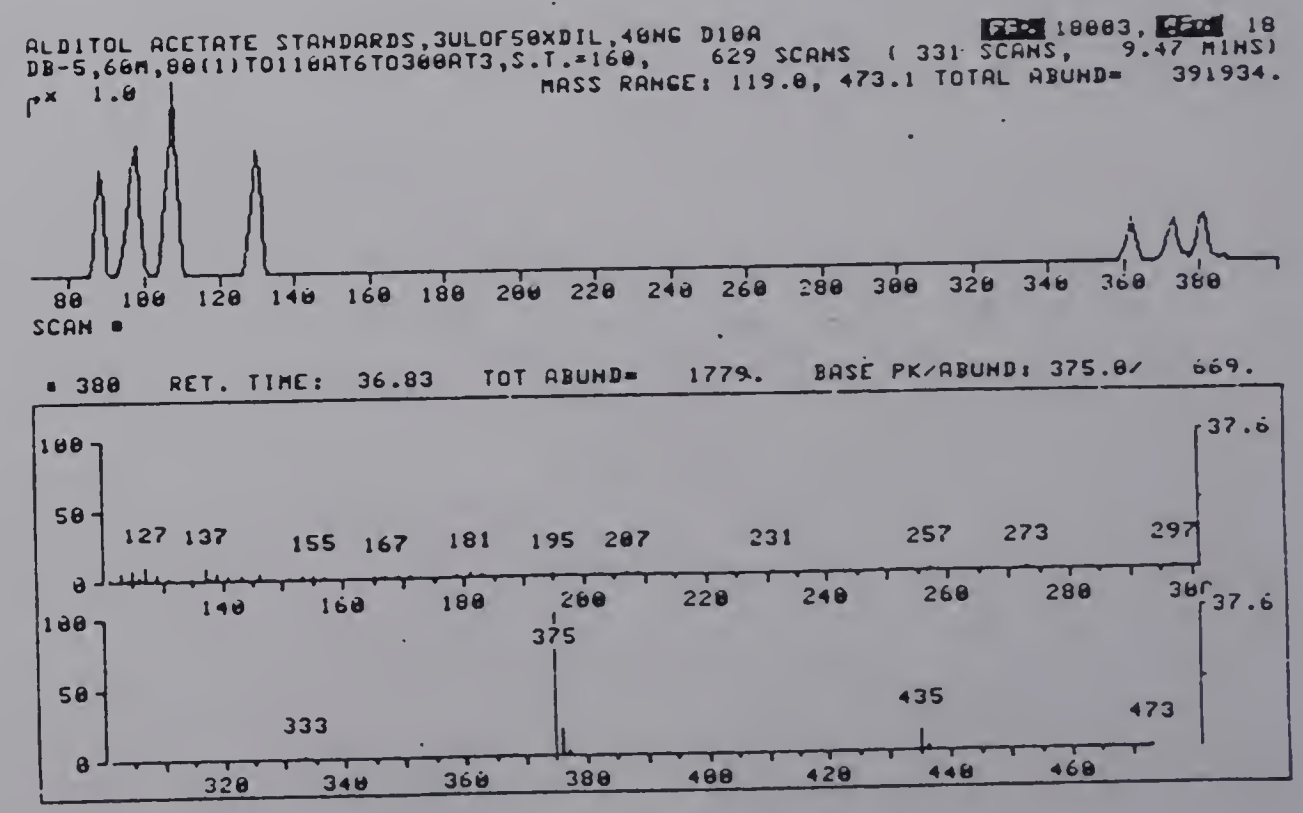


Figure 17F & G

Figure 18a

FID traces of the alditol acetate derivatives in 0.6ul injections of (A) 5.032mg FDS-W-no NO_3 and (B) 5.026mg FDS-W-NO_3 samples (in 100ul of chloroform) hydrolyzed for 1 hour at 100 C in 2 N TFA. Commercially prepared 3% OV-225 on 80-100 mesh supelcoport was used as column packing. 3/3/86

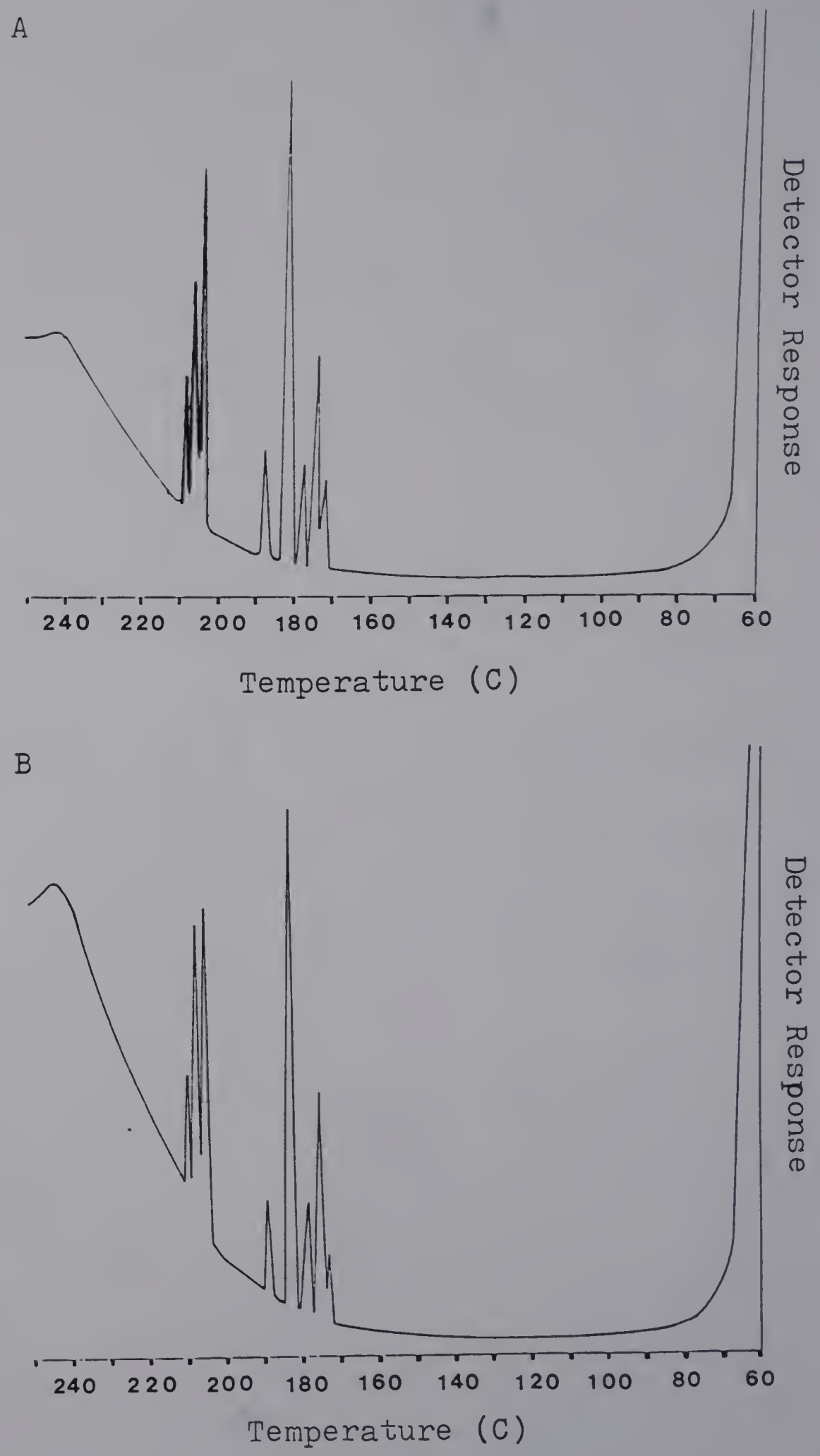
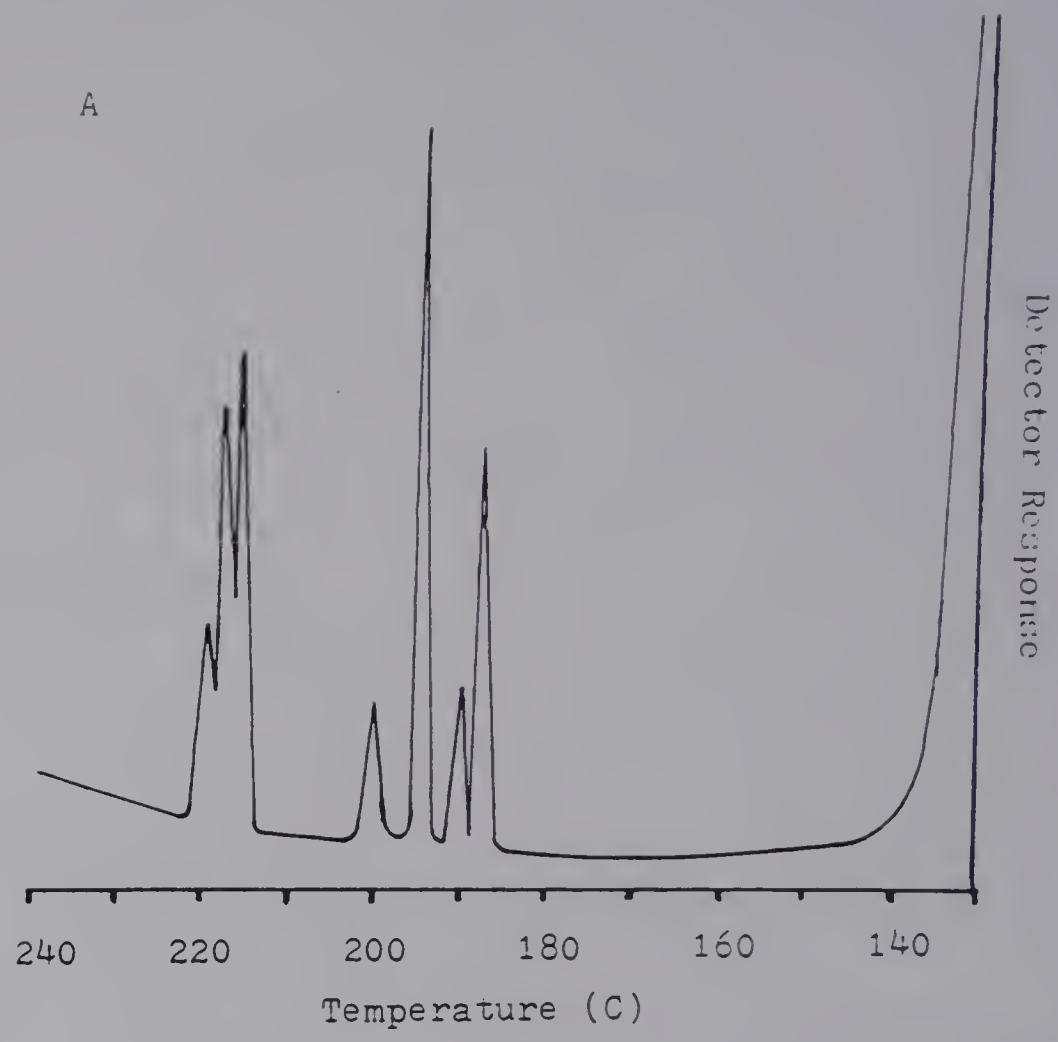

A
Detector Response
240 220 200 180 160 140 120 100 80 60
Temperature (C)
B
Detector Response
240 220 200 180 160 140 120 100 80 60
Temperature (C)

Figure 18a

Figure 18b

FID traces of the alditol acetate derivatives in 0.6ul injections of (A) 5.026mg FDS-W-NO_3 and (B) 5.032mg FDS-W-no NO_3 samples (in 100ul of chloroform) hydrolyzed for 1 hour at 100 C in 2 N TFA. 3/12/86


A
240
220
200
180
160
140
Temperature (C)
Detector Response

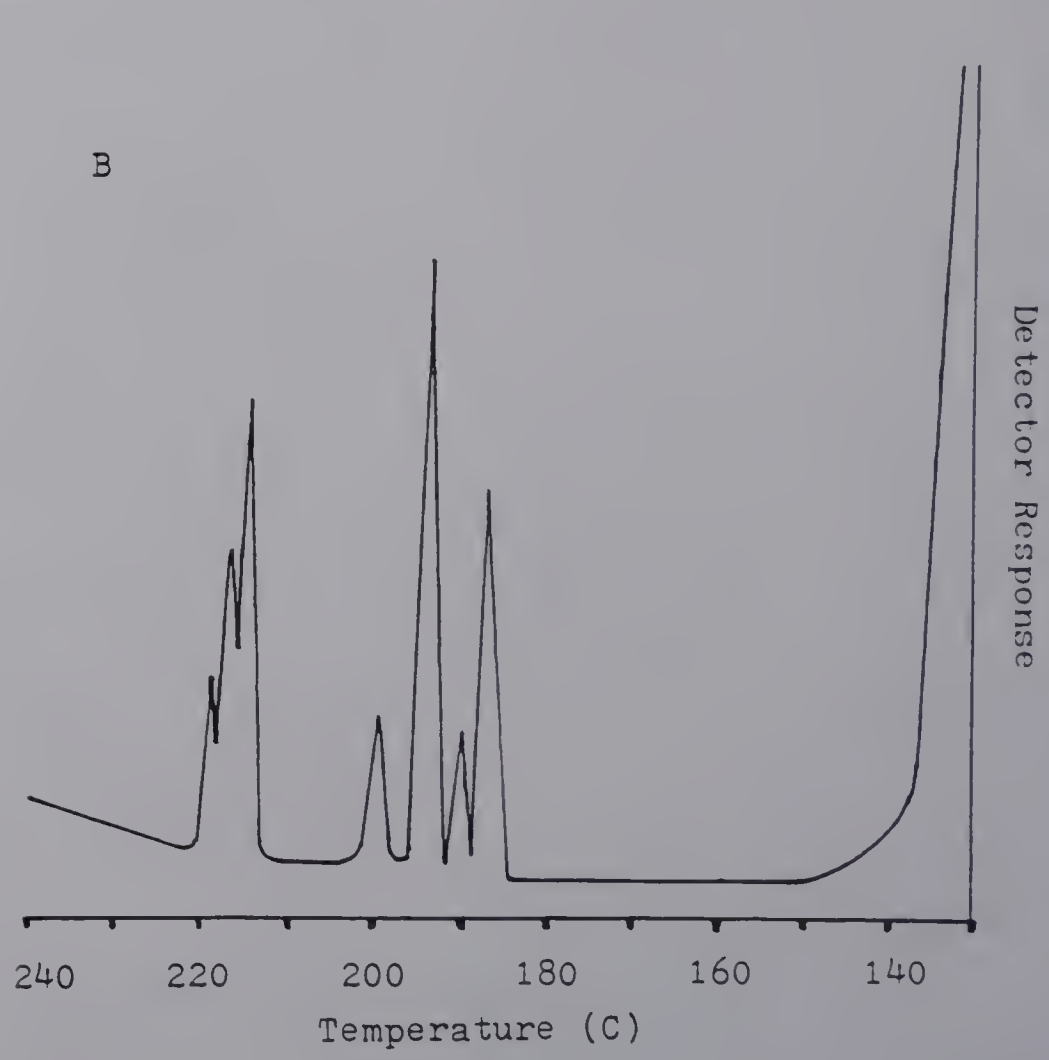

B
240
220
200
180
160
140
Temperature (C)
Detector Response

Figure 18b

Figure 19

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 5.04mg FDS-W-no NO_3 sample (in 100ul of chloroform) hydrolyzed for 35 minutes at 100 C in 2 N TFA. 3/12/86

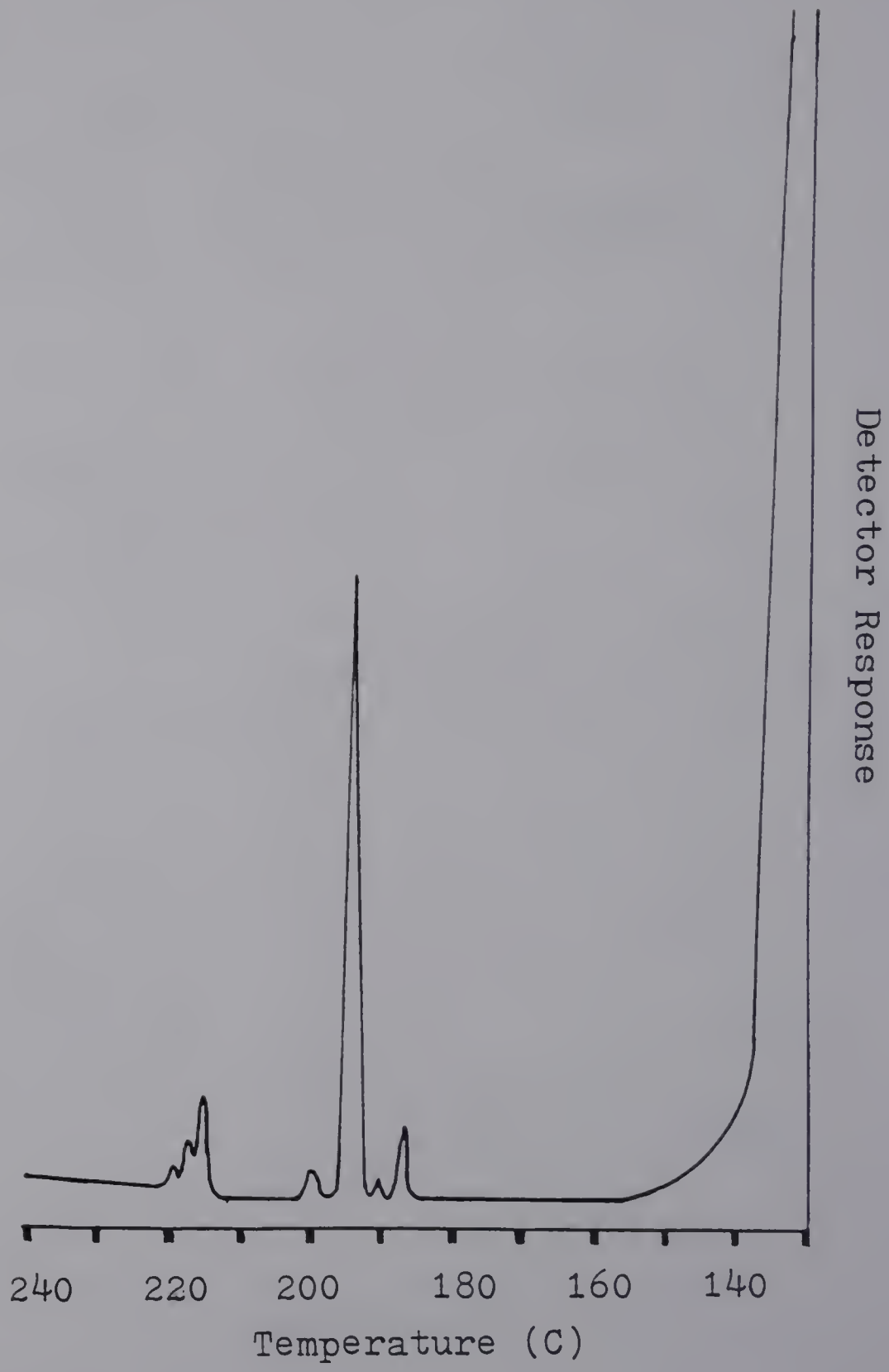


Figure 19

Figure 20

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 4.98mg FDS-W-no NO_3 sample (in 100ul of chloroform) hydrolyzed for 45 minutes at 100 C in 2 N TFA. 3/12/86

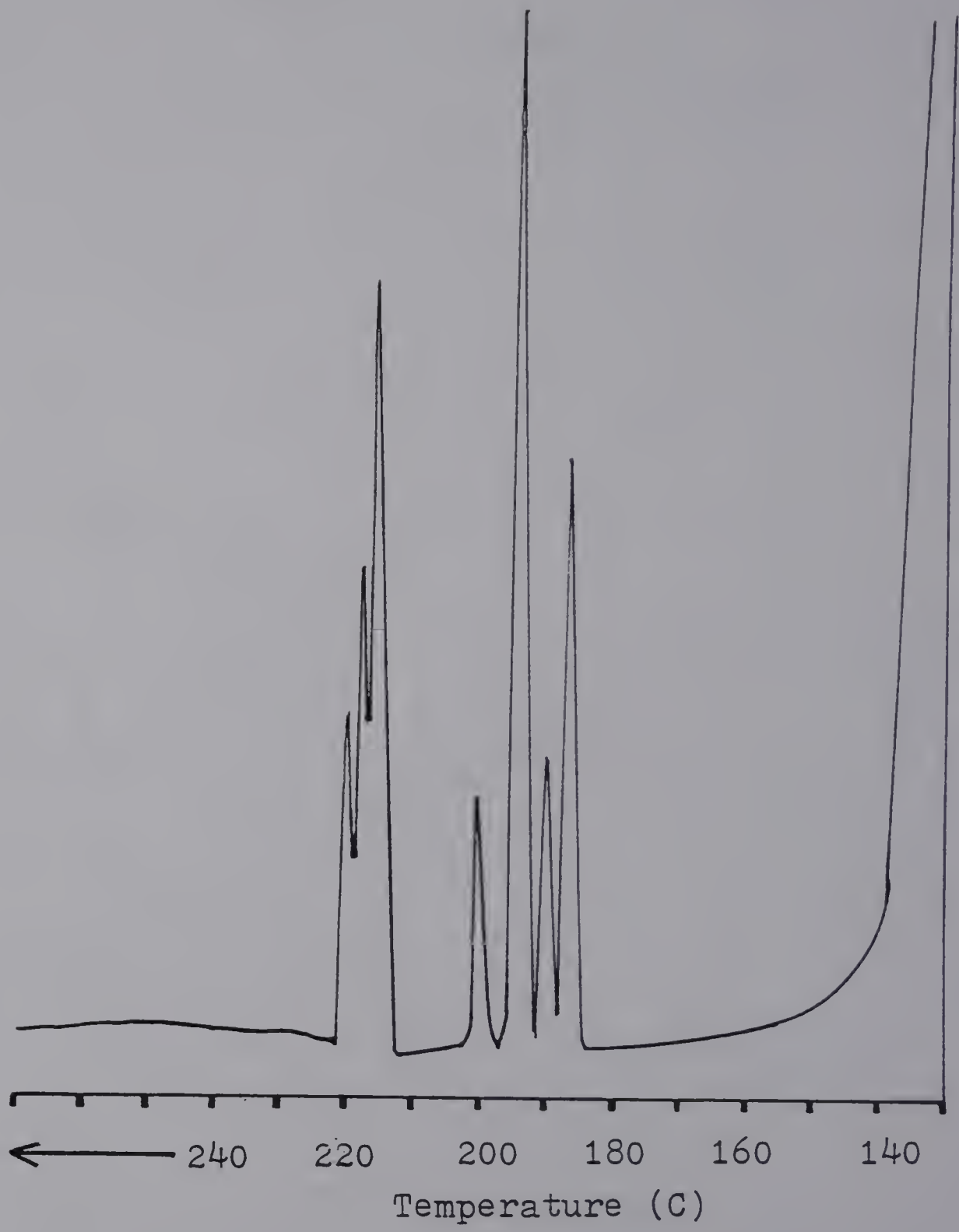


Figure 20

Figure 21

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 4.99mg FDS-W-no NO_3 sample (in 100ul of chloroform) hydrolyzed for 1 hour at 100 C in 2 N TFA. 3/12/86

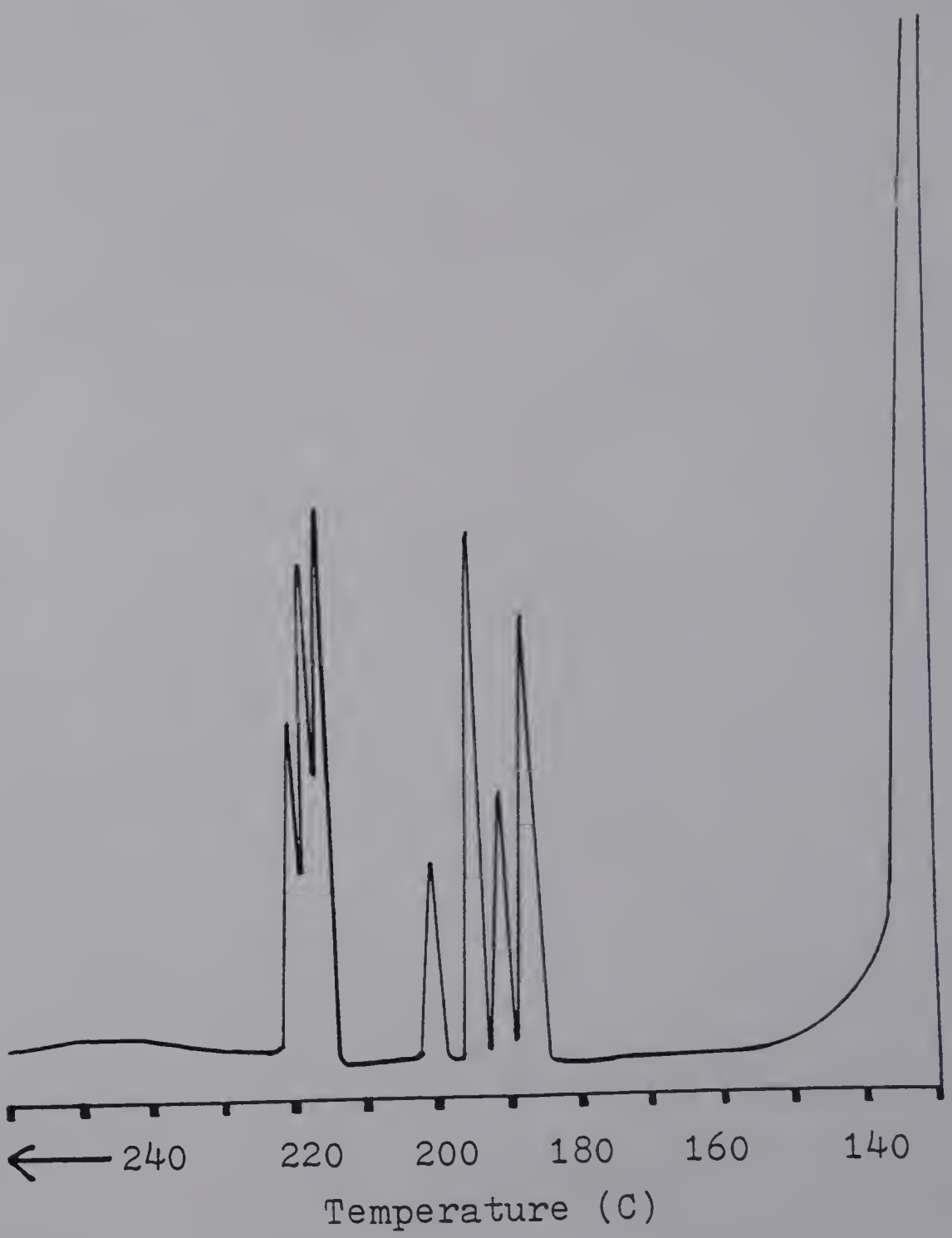


Figure 21

Figure 22

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 5.07mg FDS-W-no NO_3 sample (in 100ul of chloroform) hydrolyzed for 6 hours at 100 C in 2 N TFA. 6/4/86

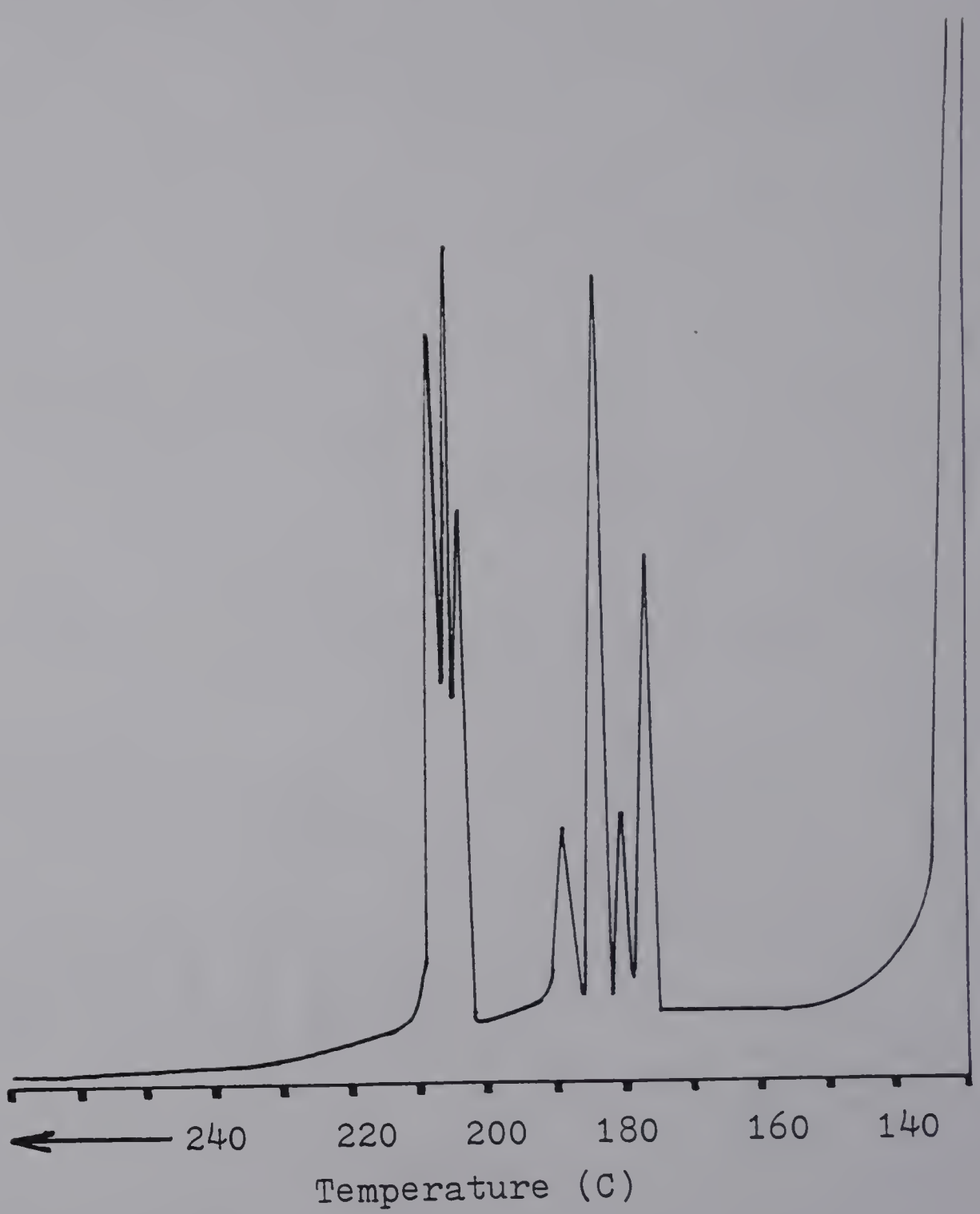


Figure 22

Figure 23

FID trace of the alditol acetate derivatives in a 0.6ul injection of a 2.69mg FDS-W-NO_3 sample (in 50ul of chloroform) hydrolyzed for 5 hours at 100 C in 1 N H_2SO_4. 7/3/86

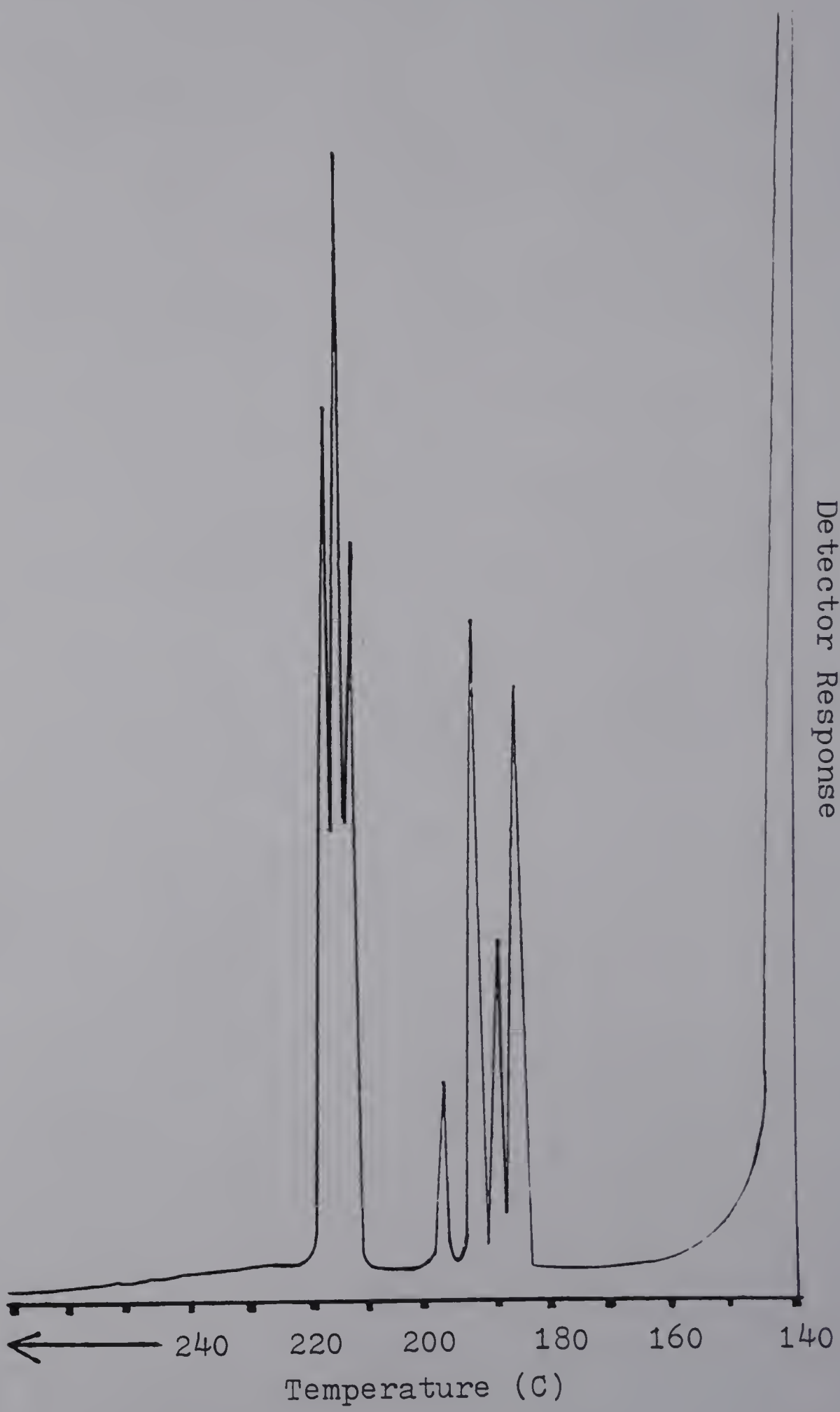


Figure 23

Figure 24

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 2.77mg FDS-W-NO_3 sample (in 50ul of chloroform) hydrolyzed for 6 hours at 100 C in 1 N H_2SO_4. 6/26/86

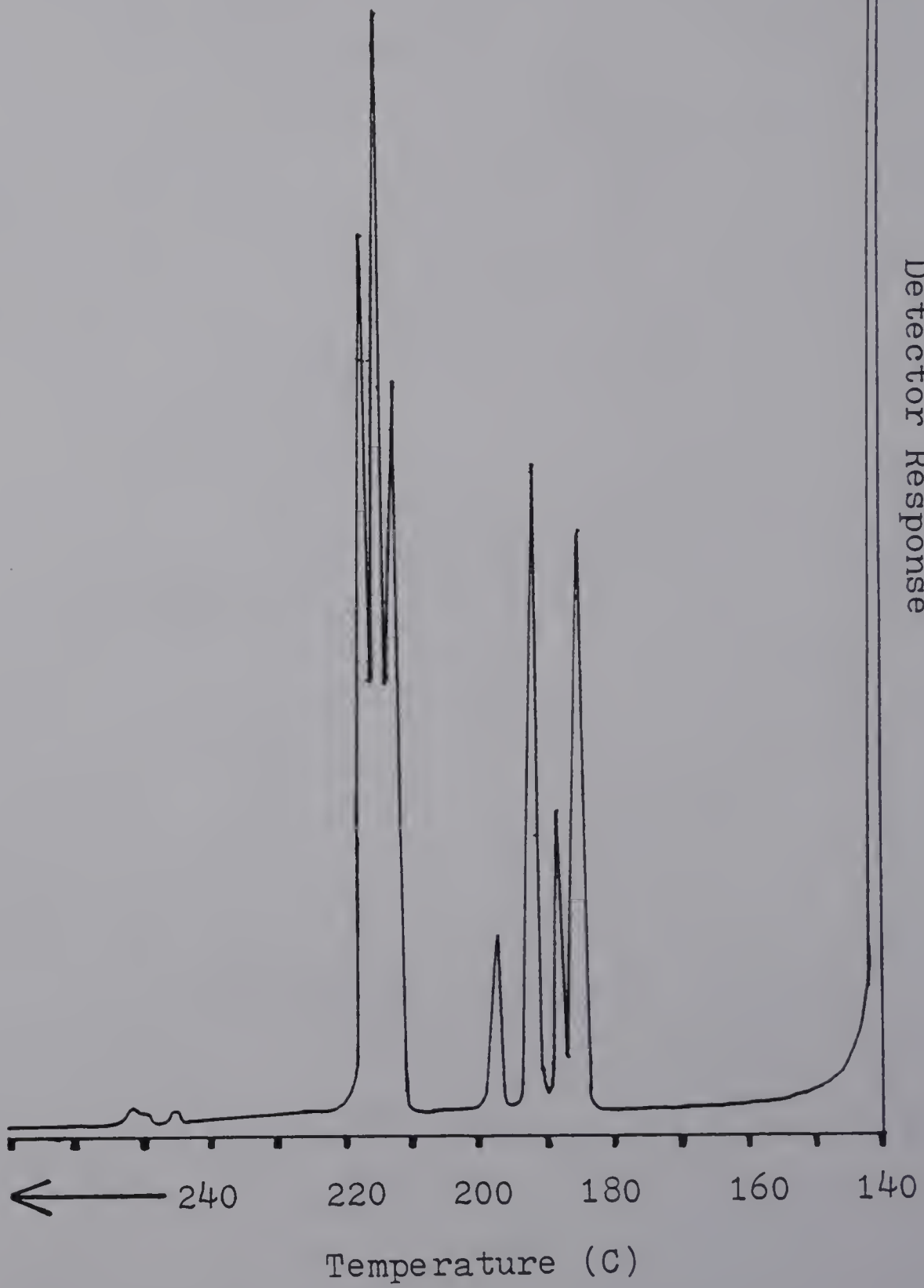


Figure 24

Figure 25

FID trace of the alditol acetate derivatives in a 0.6ul injection of a 2.58mg FDS-W-NO_3 sample (in 50ul of chloroform) hydrolyzed for 10 hours at 100 C in 1 N H_2SO_4. 7/3/86

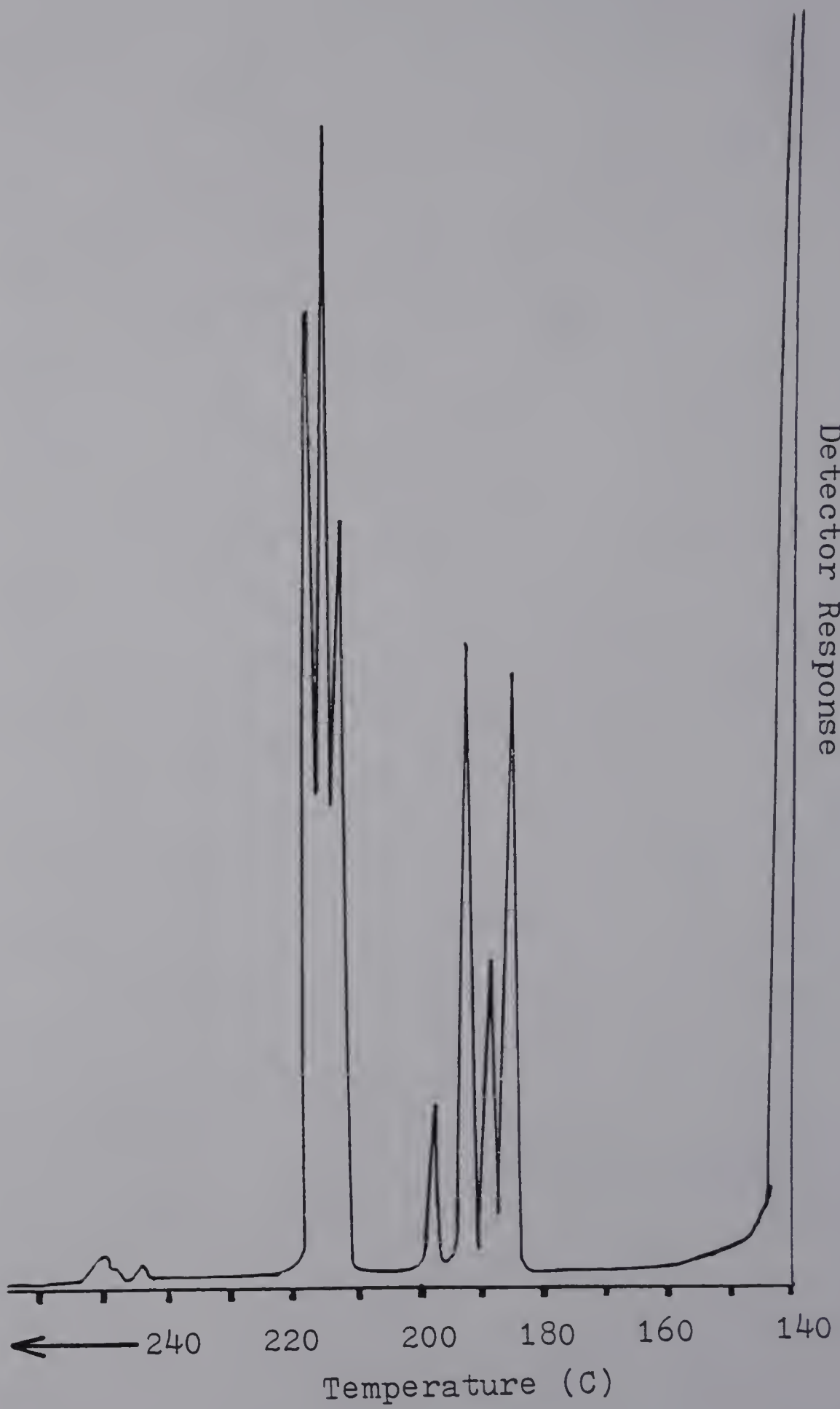


Figure 25

Figure 26

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 2.62mg FDS-W-NO_3 sample (in 50ul of chloroform) hydrolyzed for 15 hours at 100 C in 1 N H_2SO_4. 6/26/86

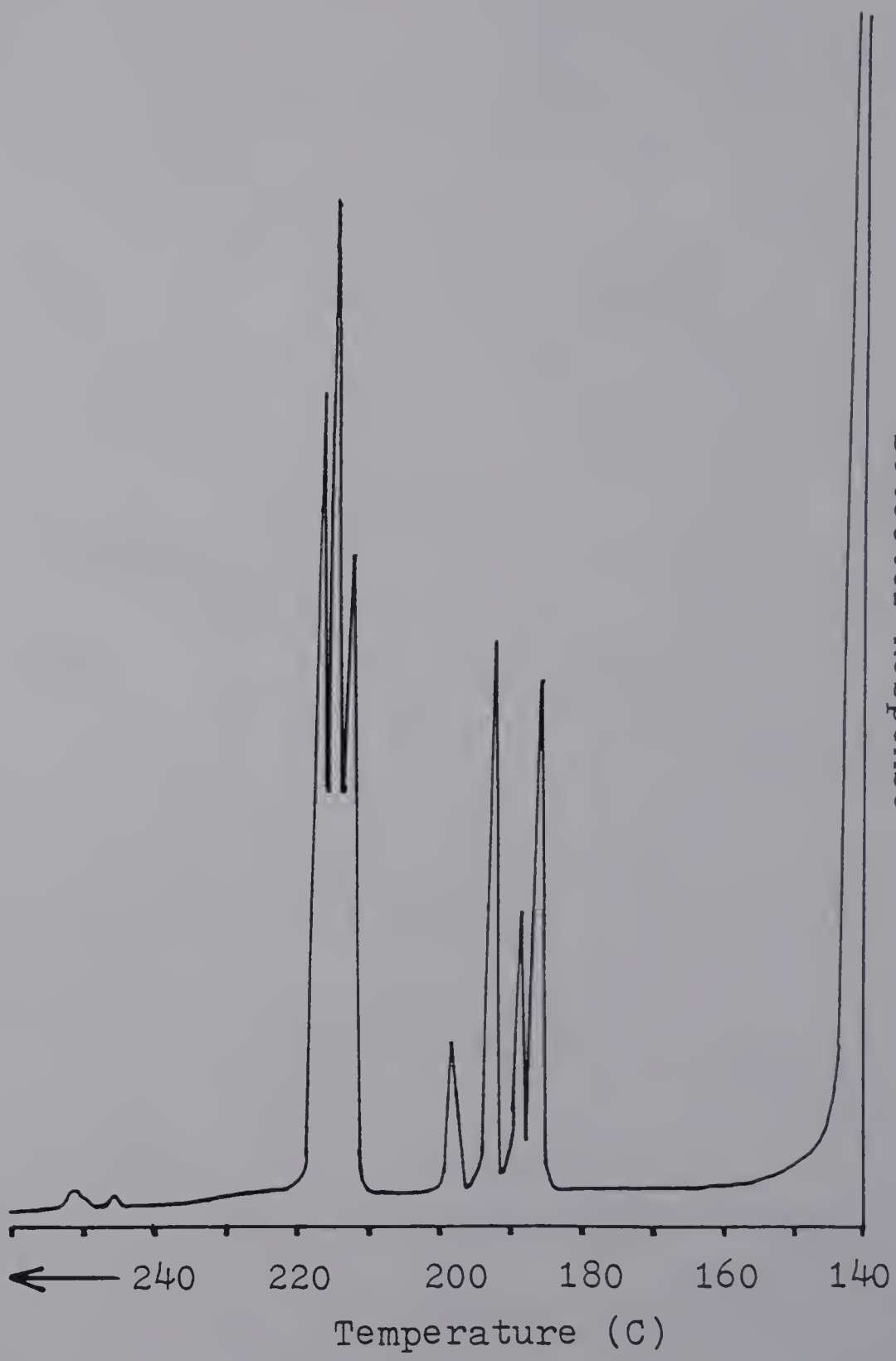


Figure 26

Figure 27

FID trace of the alditol acetate derivatives in a 0.6ul injection of a 2.57mg FDS-W-no NO_3 sample (in 50ul of chloroform) hydrolyzed for 5 hours at 100 C in 1 N H_2SO_4. 7/3/86

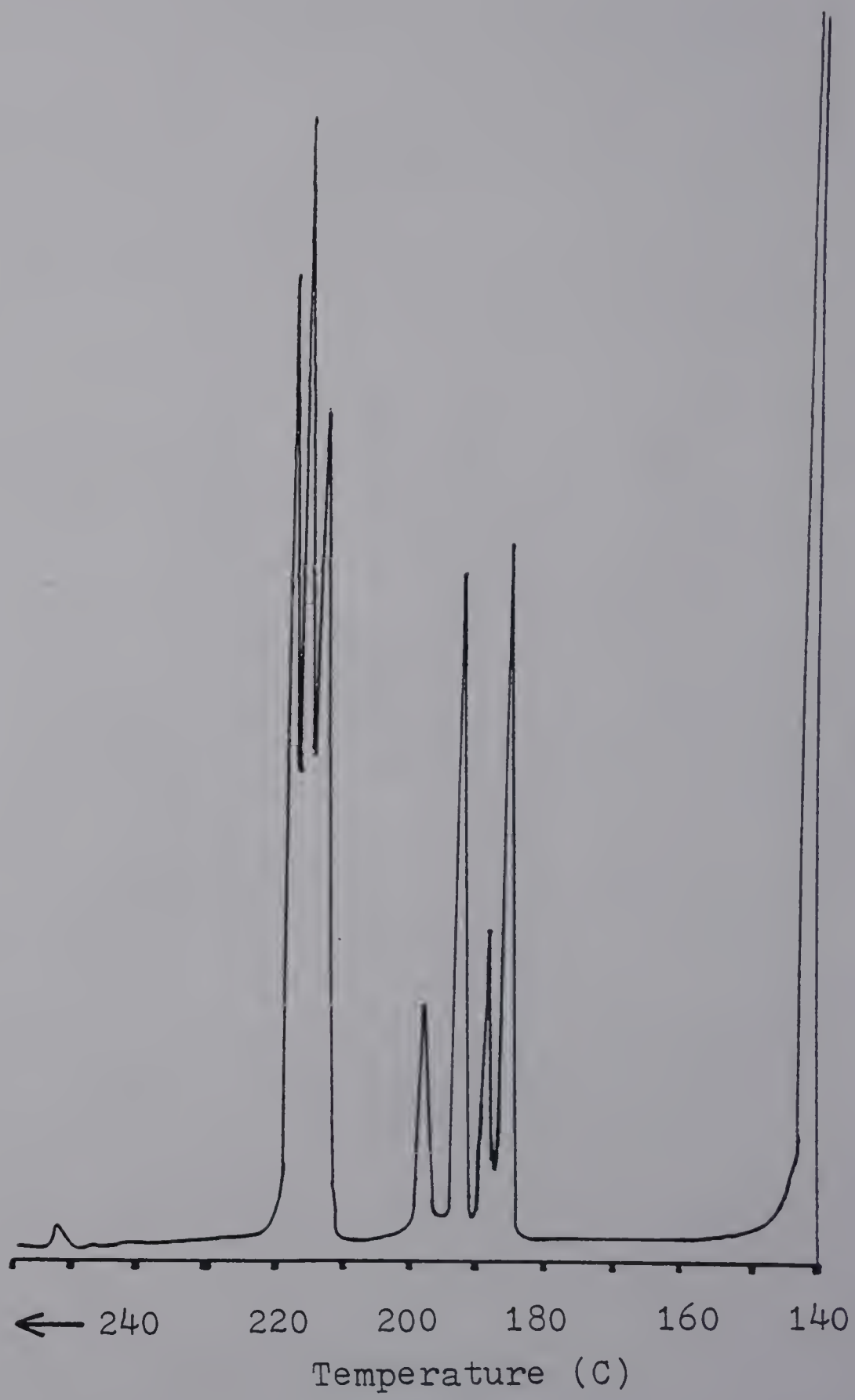


Figure 27

Figure 28

FID trace of the alditol acetate derivatives in a 0.6ul injection of a 2.61mg FDS-W-no NO_3 sample (in 50ul of chloroform) hydrolyzed for 10 hours at 100 C in 1 N H_2SO_4. 7/3/86

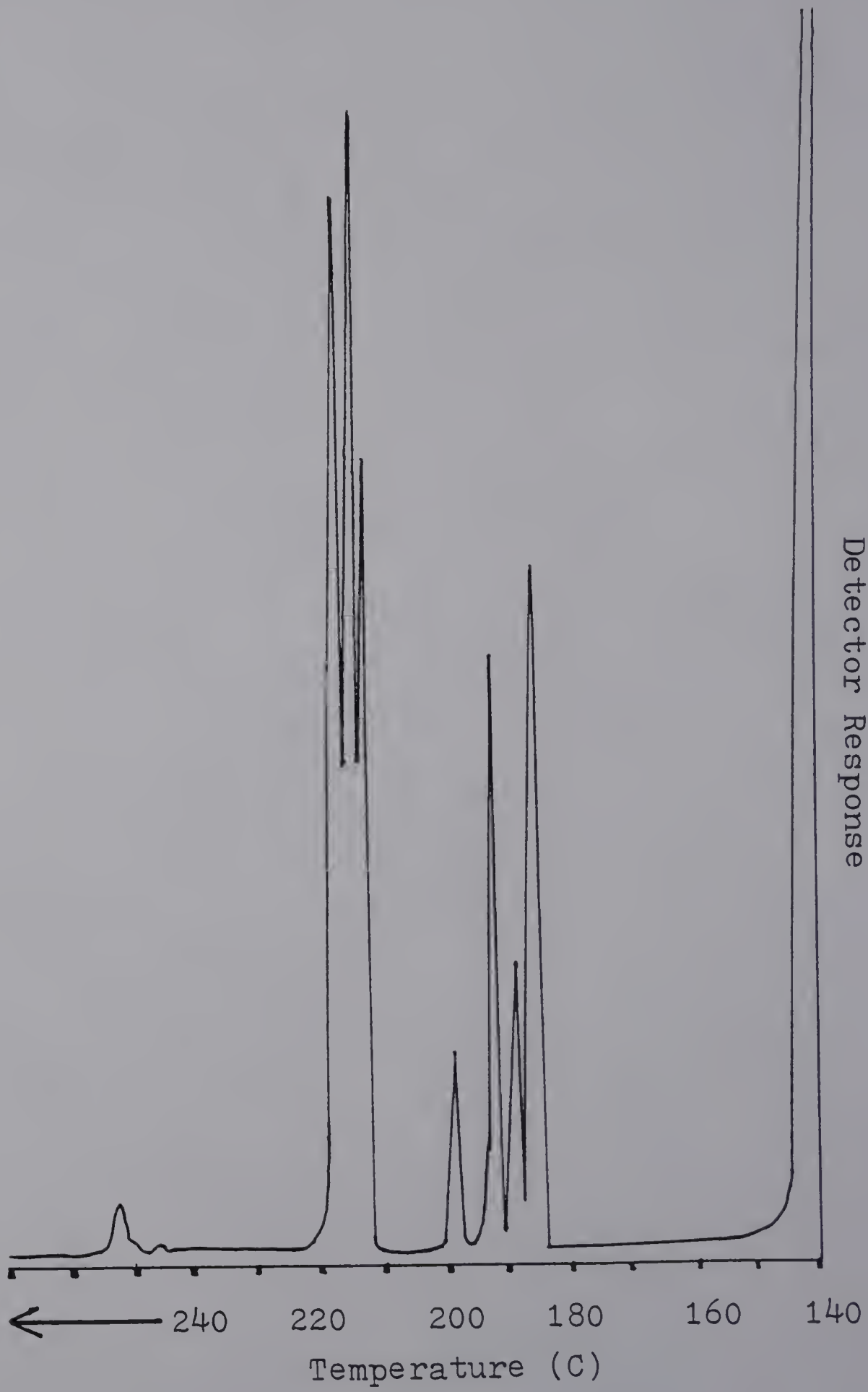


Figure 28

Figure 29

FID trace of the alditol acetate derivatives in a 0.5ul injection of a 2.58mg FDS-W-no NO_3 sample (in 50ul of chloroform) hydrolyzed for 15 hours at 100 C in 1 N H_2SO_4. 6/19/86

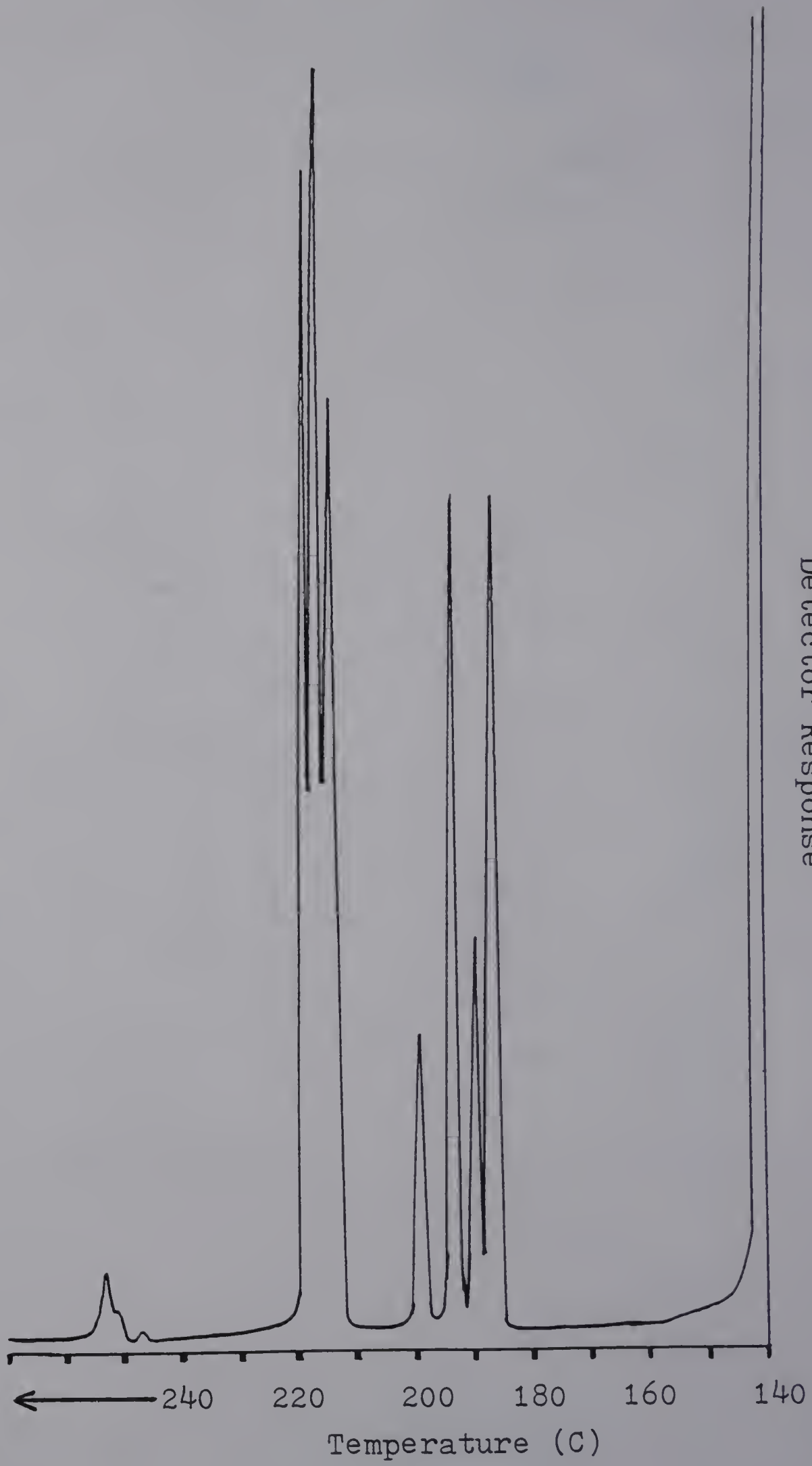


Figure 29

Figure 30

FID trace of the alditol acetate derivatives in a 1.0ul injection of a 2.62mg FDS-W-no NO_3 sample (in 50ul of chloroform) hydrolyzed for 20 hours at 100 C in 1 N H_2SO_4. 6/19/86

Figure 30

Figure 31

FID trace of a 5.02mg FDS-W-no NO_3 sample hydrolyzed for 3 hours at 100 C in 2 N TFA, showing interference with TFA during uronic acid derivatization procedures 3/31/86.

Figure 31

Figure 32

FID trace of the alditol acetate derivatives in a 1.0ul injection of a 2.60mg FDS-W-no NO_3 sample (in 50ul of chloroform) hydrolyzed for 1 hour at 100 C in 1 N H_2SO_4. 6/19/86

Figure 32

Figure 33

FID trace of the alditol acetate derivatives in a 0.5ul injection of a standard solution containing 3 ug/ul each of the following sugars: rhamnose (1), 2-O-methyl xylose (2), arabinose (3), xylose (4), mannose (5), Galactose (6), glucose (7), mannuronic lactone (8), glucuronic acid (9) and galacturonic acid (10).

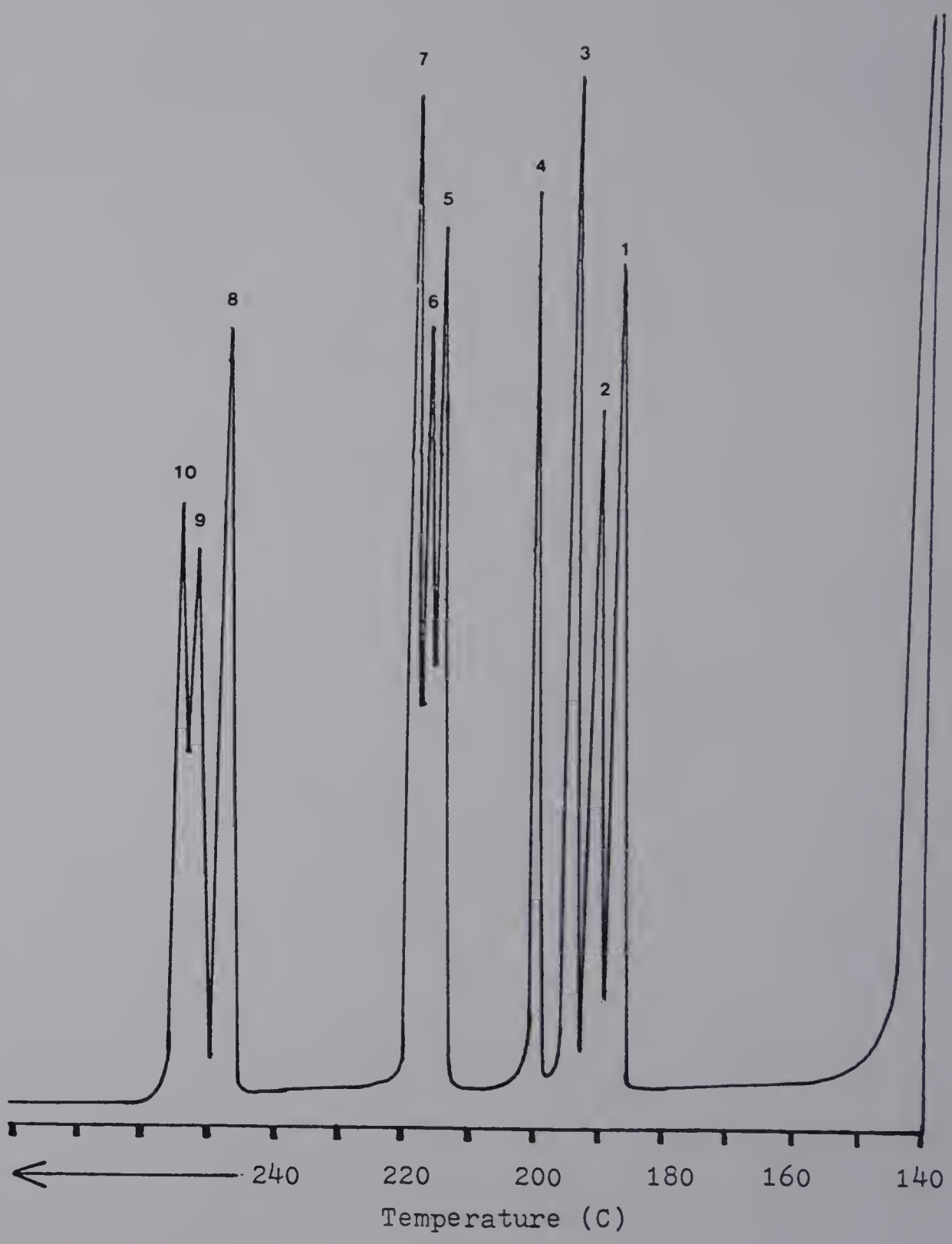

10
9
8
7
6
5
4
3
2
1
Detector Response
240
220
200
180
160
140
Temperature (C)

Figure 33

Figure 34

The diffusion pattern of the dextran polymer solutions based on the corrected uptake values ($R^{\bar{w}}$) and the molecular radii of each dextran.

Figure 34

Figure 35

The adsorption of cadmium ions by sheath investments collected from cultures grown with (NO_3) and without (no NO_3) a combined nitrogen source.

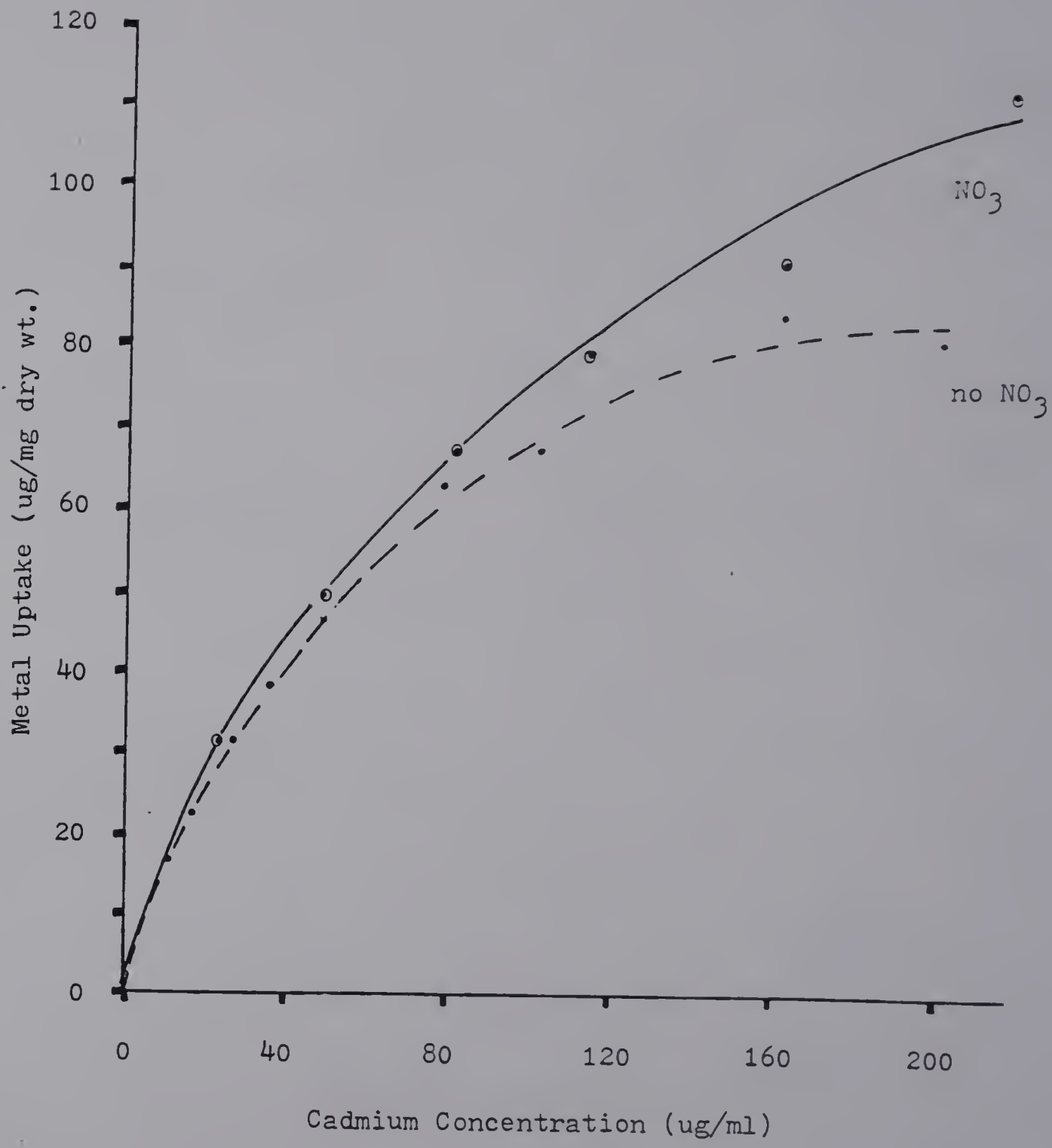

120
100
80
60
40
20
0
Metal Uptake (ug/mg dry wt.)
NO_3
no NO_3
0
40
80
120
160
200
Cadmium Concentration (ug/ml)

Figure 35

CHAPTER FIVE

Discussion

5.1 Structural Integrity

A procedure for the physical removal of the sheath investment of Gloeothece ATCC 27152 was developed. Isolated material was considered to be collectively homogeneous, based upon the absence of whole cells and the negligible phosphorus (.06%) and lipid (.3-.5%) content. However, the slow growth rate of the test cultures and the high water content of the sheath investment made the acquisition of even a few milligrams (dry weight) an arduous and often tedious task. Despite these trammels, satisfactory amounts of sheath investment material were obtained, which were quite suitable for chemical analyses and heavy metal binding studies. Microscopic observation indicated that sheath investment preparations were composed of ruptured investments. The resiliency of this material never waned during rotary ball milling, centrifugation

or lysozyme and SDS treatments. Even after several years, lyophilized material reclaimed its extended conformation upon rehydration.

The structural integrity of the sheath investment of Gloeothece ATCC 27152 is perhaps the most discerning feature distinguishing this external layer from glycocalyces and slime layers. Liberation of sheath investments into the culture media occurred only upon an external disruption of cell aggregates. Consequently, cultures of Gloeothece ATCC 27152 were virtually free of extracellular polysaccharide material, a trait very uncharacteristic of glycocalyces and slime layers.

The continuative nature of the sheath investment structure appeared to be a consequence of the melding of a variety of constituents. Chemical analyses showed that lyophilized sheath investments were composed of an acidic heteropolysaccharide, substituted with pyruvic acid, sulfate and O-acetyl groups. A triad of uronic acids, mannuronic, glucuronic and galacturonic acid, together with rhamnose, 2-O-methyl xylose, xylose, mannose, galactose and glucose, comprised the sugar constituents of the sheath investment. A 6-10% protein content remained after isolated sheath investments were

treated with lysozyme and SDS. Amino acid and electrophoretic analyses confirmed the presence of a complex protein with 2 major subunits of 51,000 and 56,000 molecular weight.

The arrangement of these components into polysaccharide chains establishes a sac-like, hygroscopic envelope, with molecular seiving capabilities for particles with diameters in excess of 16nm. Together with a general metal binding capacity of 80-110 ug Cd/mg (dry weight), the sheath investment provides a physical barrier to environmental components, selectively adsorbing as well as restricting their access to the cell.

5.2 Variability of Sheath Investment Components

Cultivation of Gloeothece ATCC 27152 without a combined nitrogen source induced the organism to fix atmospheric nitrogen. Chemical characterization of sheath investments, produced by cultures grown with and without sodium nitrate, revealed several interesting differences in the proportion of the various components detected.

While qualitative differences between the components

comprising both sheath investment preparations were lacking, quantitative dissimilarities were abundant. Total carbohydrate, O-acetyl groups and protein were 24%, 26%, and 42% greater for FDS-W-NO_3 than FDS-W-no NO_3 material, respectively. The water content of the sheath gel prior to lysozyme and SDS treatments was greater for sheath investments collected from cultures fixing atmospheric nitrogen. Microscopic examination of both preparations revealed that sheath investments collected from cultures grown without a combined nitrogen source were more refractive and voluminous compared to investments collected from cultures grown with sodium nitrate (Figures C & D).

The chemical composition of the sheath investment preparations is summarized in Table 21. The total uronic acid content for both preparations were considered to be the same, based upon gas chromatography results and due to the inability of the colorimetric assay to accurately detect mannuronic acid. While the list of constituents appears to be complete for sheath investments collected from cultures assimilating sodium nitrate, this does not seem to be the situation for sheath investments collected from cultures fixing atmospheric nitrogen. Approximately 20%

of this material was unaccountable. Perhaps the remaining proportion of sheath investment material was comprised of material yet to be identified. Malic and formic acids are common entities in bacterial external layer polysaccharides. A predominance of these constituents in the sheath investments, collected from cultures fixing atmospheric nitrogen could account for the discrepancy observed in the tallied constituents.

5.3 Metal Binding

The sheath investment of Gloeothece ATCC 27152 maximally bound 80-110 ug of cadmium ions per mg of lyophilized sheath material. Les & Walker (1983) reported a binding capacity of 53 ug Cd/mg for whole cells of Chroococcus paris. Comparison of these results would be difficult due to the nature of the test material. It is impossible to determine the extent to which the cell wall of C. paris contributed to the reported binding capacity. Additionally, chemical variations in the sheath investment of C. paris would induce changes in the metal adsorption ability, making any comparison to the sheath investment of Gloeothece ATCC 27152 inappropriate.

The proportional variation in sheath investment constituents perhaps contributed to the 27% increase in the metal binding ability of FDS-G-NO_3 material over FDS-G-no NO_3 material. Ascertaining which components were most responsible for this change in binding capacity would be difficult if not inappropriate. Alterations in the metal binding ability of the sheath investments may not be associated with the abundance of individual binding sites as much as the result of an

alteration in the polysaccharide conformation. Pockets and protrusions in the polysaccharide secondary structure may sequester and/or chelate cations with or without the aid of anionic residues (McCandles, 1981). Changes in only a few residues could have a significant influence on the structural conformation and subsequently affect the binding ability of the sheath investment (Rees, 1975).

In light of the several metal binding components detected, the potential for the accumulation of a variety of nutrients and minerals in the sheath investment would appear to be high. Electron donors such as hydroxyl, sulfate, carboxyl and peptide groups have varying affinities for metal ions (Eichhorn, 1975). Changes in their occurrence would affect the binding capacity of the material they comprise. Attenuation of the binding ability of the sheath investment, mediated directly by the cell or as an indirect consequence of environmental conditions would be an interesting aspect of Gloeothece ATCC 27152. Further research, however, is obviously required to support this possibility.

5.4 Neutral Sugars

The ratio of the neutral sugars: rhamnose, 2-O-methyl xylose, xylose, mannose, galactose and glucose, in the sheath investments collected from cultures grown with and without a combined nitrogen source were 3:2:1:6.5:10:8 and 3.5:1.5:1:6:9:8, respectively (hydrolysis was with 1 N H_2SO_4 for 10 hours ar 100 C). While the proportion of neutral sugars for both sheath investment preparations were relatively equivalent under these conditions, hydrolysis for only 5 hours, revealed a greater proportion of mannose, galactose and glucose, in FDS-no NO_3 over FDS-NO_3 material. Perhaps the greater protein content of FDS-NO_3 material served to stabilize the sheath investment, making it more resistant to acid hydrolysis than sheath investments collected from cultures fixing atmospheric nitrogen.

Short term hydrolysis of FDS-no NO_3 material revealed a variation in acid lability among the sugar residues. Relative proportions of rhamnose, 2-O-methyl xylose, xylose and mannose were greatest after hydrolysis in 2 N TFA for 35 minutes at 100 C. The maximal liberation of galactose and glucose residues required much longer hydrolysis times.

If the heteropolysaccharide is composed of repeating units, it is likely that the backbone is primarily made of glucose and galactose residues, with mannose in a more external location (i.e. side branches). Rhamnose, xylose and 2-O-methyl xylose would likely be located as terminal residues based upon their susceptibility to mild acid hydrolysis. Due to their lipophilic nature, rhamnose and 2-O-methyl xylose could be internally located on the cellular side of the sheath investment. Lipophilic sugar residues would be appropriate for anchoring the sheath investment to the outer cell membrane during early assembly of nucleotide sugars.

5.5 Uronic Acids

While differences in the ratio of neutral sugars were negligible, the proportion of mannuronic, glucuronic and galacturonic acids varied between FDS-NO_3 and FDS-no NO_3 material. When Gloeothece ATCC 27152 assimilated combined nitrogen, FDS-NO_3 material possessed a ratio of 1:1:2 for the previously mentioned uronic acid triad. During nitrogen fixing conditions, however, the amount of mannuronic acid decreased in relation to glucuronic and galacturonic acids, yielding a ratio of 1:3:4.

Gas chromatographic analyses indicated that the total uronic acid content was relatively similar for both sheath investment preparations. However, colorimetric analyses revealed a predominance in total uronic acid content for FDS-W-no NO_3 material. The lower proportion of mannuronic acid in sheath investments collected from cultures fixing atmospheric nitrogen could account for the higher uronic acid content detected with the m-phenyl phenol assay. The molar extinction coefficient for mannuronic acid is much less than glucuronic and galacturonic acids (Blumenkrantz & Asboe-Hansen, 1973). Consequently,

material with more mannuronic acid would appear to possess a lower total uronic acid content. If differences in total uronic acid content exist between sheath investment preparations, it would appear to be only minimal.

Chromatograhic analyses accounted for only 30% of the total uronic acid content detected by the colorimetric assay. Prolonged acid hydrolysis with 1 N sulfuric acid may have caused decomposition of liberated uronic acid residues. If acid hydrolysis was extended to longer periods, more uronic acid residues would be liberated but their accumulation would be prevented due to continued acid degradation. Consequently, no more than 2% of the total uronic acid content was detectable at one time. Colorimetric analysis utilized sodium tetraborate in conjunction with concentrated sulfuric acid to achieve maximum hydrolysis of sheath investment material. Sodium tetraborate interacted with free uronic acids, thereby shielding them from further acid degradation (Blumenkrantz & Asboe-Hansen, 1973). Problems associated with excessive barium carbonate neutralization prevented the use of stronger acid concentrations during chromatographic analyses.

Consequently, quantitative estimates of the total uronic acid content were derived from the colorimetric assay while gas chromatography provided a means for the identification of the individual residues.

It might be advantageous if, in the future, hydrolysis of acidic polysaccharides (for GC analysis) included sodium tetraborate. Assuming the salt does not interfere with derivatization procedures, liberated uronic acid residues may be shielded from further degradation during prolonged acid hydrolysis.

5.6 Sulfate Content

Sheath investments collected from cultures of Gloeothece ATCC 27152, grown with and without $NaNO_3$, contained 5.15% and 5.30% sulfur, respectively. This corresponded to a theoretical sulfate content of 15.5% and 15.9%, respectively. Acid hydrolysis at 100 C, in 0.5 N HCl, for 1 hour, detected 56% and 83% of the total theoretical sulfate content for FDS-G-NO_3 and FDS-G-no NO_3 material, respectively. No sulfate was detected under weaker hydrolytic conditions.

Based upon comparable total sulfur values, the total sulfate content of the sheath investment preparations should be at least proportional. The lower sulfate content detected in FDS-G-NO_3 material could be attributed to varying rates of hydrolysis between sulfate esters located at different positions within the sugar ring as well as throughout the polysaccharide chain. According to Rees (1963), the lability of sulfate groups to acid hydrolysis is dependent upon their attachment to primary or secondary hydroxyl sites of a sugar ring. Sulfate esters involving the oxygen of a primary hydroxyl group are most resistant, with a half life exceeding 1.5 hours when hydrolyzed in 0.25 N HCl at 100 C. Sulfate groups esterified to an axial

secondary hydroxyl group possessed 1-1.5 hour half lives, while those attached in the equatorial position possessed half lives ranging from 0.1-0.4 hours. Therefore, the lower sulfate content detected in FDS-G-NO_3 material could be attributed to the greater occurrence of sulfate esters at the primary hydroxyl positions over the more labile secondary hydroxyl sites. Sheath investment preparations hydrolyzed for 28 hours at 100 C, in 1 N HCl, yielded values comparable to the theoretical sulfate content. Sulfate esters resistant to the milder hydrolytical conditions were released by this harsher treatment.

Apart from the dissimilarity in sulfate lability between sheath investment preparations, the discovery of sulfate groups in the external layer of a cyanobacterium is one of more far reaching significance. While sulfate containing extracellular polysaccharides are common among a number of eucaryotic organisms, their inclusion in the procaryotic domain has yet to be documented. Sulfated sugars occur in the polysaccharides of fresh water and marine algae as well as in animal cartilage and connective tissue. The sulfate anion in these polysaccharides serves to maximize the contact with water molecules (Rees, 1972).

The abundance of sulfate esters among the various algal and animal acidic polysaccharides regulates their physical temperament. As the proportion of sulfate groups increases, the gelling ability of the material decreases. The anionic character imparted by the sulfate esters also serves in an ion exchange capacity and/or in sequestering certain cations (McCandles, 1981).

While the discovery of sulfated sugars among cyanobacteria would certainly be unique, it would not necessarily be one of total surprise. Cyanobacteria occupy a very strategical position in the tree of evolution. Classified as procaryotic in nature, this transitional group possesses features characteristic of eucaryotic organisms as well: differentiation in cell structure (i.e. heterocysts), oxygenic photosynthesis involving photosystem I & II and chlorophyll-a. Sulfated sugars have never been detected among bacterial polysaccharides. Their occurrence in the cyanobacteria, however, would lend further credibility to the transitional character these organisms possess regarding evolutionary development.

5.7 Protein Content

The protein content of the sheath investment, collected from cultures fixing atmospheric nitrogen was 6%. Comparative analyses showed that sheath investments collected from cells assimilating $NaNO_3$ contained 42% more protein than those collected from cultures fixing atmospheric nitrogen. Considering the large amount of energy required for nitrogen fixation, the decrease in protein detected in FDS-W-no NO_3 material was understandable.

It became apparent that the protein detected in the sheath investment of Gloeothece ATCC 27152 was an integral component and not an artifact of isolation procedures. The authenticity of this conclusion was sustained by the low abundance of protein subunits observed via electrophoresis. If the preparations were contaminated with membrane proteins, a greater number of protein bands would have undoubtedly been observed. Similarly, the persistance of the protein component after lysozyme and SDS treatments suggested that a covalent association occurs with the acidic polysaccharide network of the sheath investment.

The structural and functional aspects of the sheath

investment protein remains uncertain. Structurally, the protein could serve to anchor the sheath investment to the cell surface during assembly. Upon separation, the protein appears to be maintained in the sheath investment and perhaps contributes to its structural strength and metal binding ability. While experimental results favor a more complex protein, peptide linkages may also occur, stabilizing the polysaccharide network, much like that which occurs in peptidoglycan. Enzymatically, the protein could function during nucleotide sugar assembly of the sheath investment or serve as an epimerase, inducing changes in polysaccharide conformation (i.e. desulphation of sulfated sugars).

If one component could account for the dissimilarity in metal binding capacity observed between the sheath investment preparations, the greater protein content of FDS-G-NO_3 material would be a likely possibility. Amino acids are prime candidates for binding metal ions (Eichhorn, 1975). Proteinaceous exudates from cyanobacteria have long been known for their significant metal binding abilities (Fogg, 1952).

The study of the protein component should be an area of continued interest. The elucidation of this

constituent regarding the assembly, structural integrity and metal binding ability of sheath investments would be a significant contribution in the area of external layer research. It would be interesting to learn if the sheath investment of Gloeothece ATCC 27152, grown without a combined nitrogen source (fixing atmospheric nitrogen), possessed a stonger binding affinity for Fe and Mo, two elements essential for the nitrogenase enzyme system. Changes in the occurrence of certain metal binding components in the sheath investment could selectively regulate the extent to which these metal ions are adsorbed.

CHAPTER SIX

Conclusion

The characterization of the sheath investment of Gloeothece ATCC 27152 has touched upon many facets worthy of continued interest. Future research should be directed at the inherent variability that may exist in the composition of sheath investments collected from different cultures but grown under similar conditions. Only then can dissimilarities observed in sheath investments, cultured under different culture conditions be considered as adaptive responses to environmental stimuli. The origin of the proteinaceous component of the sheath investment of Gloeothece ATCC 27152 remains unknown. At this time a structural or functional role could be equally probable. The presence of sulfated sugars in the procaryotic domain is an intriguing proposition. It would be interesting to learn the extent to which these groups occur among cyanobacterial external layers. A more thorough study should be conducted on the heavy metal binding capacity

of the sheath investment of not only Gloeothece ATCC 27152 but of other sheath investment producing cyanobacteria as well. Comparison of the chemical composition of sheath investments to the degree to which they bind metal ions would enlighten our knowledge of metal-polysaccharide complex formation. If Gloeothece ATCC 27152 is capable of selectively regulating the binding affinity and capacity of its sheath investment, this would be an uncommon example of cellular regulation at the procaryotic level.

While the majority of this work could be considered preliminary, a method for the removal and isolation of the sheath investment, as well as a comprehensive survey of its chemical constituents, has been offered. Despite the abundance of speculation, several conclusions can be drawn, regarding the structure, chemical and physical composition of the sheath investment of Gloeothece ATCC 27152:

1) The structure of the sheath investment is resilient. Its components are strategically arranged into a continuous envelope that expands to accomodate the growing population it encloses.

2) The chemical composition of the sheath investment is diverse. In addition to the neutral sugars; rhamnose, 2-O-methyl xylose, xylose, mannose, galactose glucose and the uronic acids; mannuronic, glucuronic and galacturonic acid, this heteropolysaccharide containes protein, pyruvic acid, O-acetyl and sulfate groups.

3) The presence of sulfur in the sheath investment, identified as sulfate, suggestes the occurence of sulfated sugars in a procaryotic organism, an intriguing and unique observation.

4) The protein content of the sheath investment was 6-10% and was considered to be made of primarily two complex subunits of 56,000 and 51,000 molecular weight.

5) The metal binding capacity of the sheath investment (for cadmium ions) ranged from 80 -100 ug/ mg of lyophilized sheath investment material.

6) The minimal pore size of the sheath investment was approximated at 16nm in diameter.

7) Changes in the source of nitrogen led to a physical and chemical alteration in the sheath investment. Total carbohydrate, O-acetyl groups and protein were 24%, 26% and 42% greater in sheath investments collected from cultures assimilating sodium nitrate than those fixing atmospheric nitrogen. The water content of crude sheath gel was greatest among sheath material collected from cultures fixing atmospheric nitrogen. Compared to sheath investments collected from cultures assimilating sodium nitrate, these preparations were also more voluminous and refractile when viewed microscopically. The triad of uronic acids also varied between sheath investment preparations. Mannuronic acid was more prevalent among sheath investments collected from cultures assimilating sodium nitrate. Consequently, the metal binding capacity likewise varied between sheath investment preparations. FDS-NO_3 material bound 20% more cadmium ions than FDS-no NO_3 material.

BIBLIOGRAPHY

Allen, M.M. (1968) Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4:1-3.

Allen, M.M. & Stanier, R.Y. (1968) Growth and division of some unicellular blue-green algae. J. Gen. Microbiol. 51:199-202.

Allen, H.E.; Hall, R.H. & Brisbin, T.D. (1980) Metal speciation. Effects on aquatic toxicity. Env. Sci. Tech. 14:441.

Anemiya, Y. & Nakayama, O. (1984) The chemical composition and metal adsorption capacity of the sheath materials isolated from Microcystis, cyanobacteria. JPN J. Limnol. 45: 187-193.

Bayer, M.E.; Carlemalm, E. & Kellenberger, E. (1985) Capsule of E. coli K29: Ultrastructural preservation and immunoelectron microscopy. J. of Bacteriol. 162:985-991.

Bershova, O.I.; Kopteva, Zh. P. & Tantsyurenko, E.V. (1968) The interactions between blue green algae - the causative agents of water 'bloom' and bacteria. In 'Tsvetenic' Vody Ed. Topachevsky, A.V. Naukova Dumka Kiev USSR (Russian) pp. 159-177.

Bishop, C.T.; Adams, G.A. & Hughes, E.O. (1954) A polysaccharide from the blue-green alga A. cylindrica. Can. J. of Microbiol. 11:877-885.

Biswas, B.B. (1957) Sci. Cult. 22:696-697.

Blumenkrantz, N. & Asboe-Hansen, G. (1973) New method for quantitative determination of uronic acids. Anal. Biochem. 54:484-489.

Bociek, S.M. & Welti, D. (1975) The quantitative analysis of uronic acid polymers by infrared spectroscopy. Carb. Res. 42:217-226.

Buckley, C.E. & Houghton, J.A. (1976) A study of the effects of near UV radiation on the pigment of the blue-green alga Gloeocapsa alpicola. Arch Microbiol. 107:93-97.

Butler, R.D. & Allsopp, A. (1972) Ultrastructural investigations in the Stigonematacea (Cyanophyta). Arch. Mikrobiol. 82:283-299.

Caldwell, D.E. & Caldwell, S.J. (1978) A Zoogloea species associated with blooms of Anabaena flos-aquae. Can J. Microbiol. 24:922-931.

Chang, T. (1980) Mucilage sheath as a barrier to carbon uptake in a cyanophyta, Oscillatoria rubescens D.C. Arch.Hydrobiol. 88:128-133.

Chrost, P.J. & Brzeska, D. (1978) Extracellular release of organic products and growth of bacteria in A. cylindrica (blue-green alga) culture. Acta. Microbial. Polon. 27:287-295.

Costerton, J.W.; Geesey, G.G. & Cheng, K.J. (1978) How bacteria stick. Scient. Am. 238:86-95.

Costerton, J.W.; Marrie, T.J. & Cheng, K.J. (1985) Phenomena of bacterial adhesion. In Bacterial Adhesion Ed. Savage, D.C. & Fletcher, M. Plenum Press NY & London Chap. 1 pp 7.

Crist, R.H.; Oberholser, K.; Shank, N. & Nguyen, M. (1981) Nature of bonding between metallic ions and algal cell walls. Env. Sci. Tech. 15: 1212-1217.

Daniels, S.S.L. (1980) Mechanisms involved in sorption of microorganisms to solid surfaces. In Adsorption of Microorganisms to Surfaces Ed. Bitton, G. & Marshall, K.C. Wiley-Interscience, NY. pp. 7-58.

Desikachary, T.V. (1959) Cyanophyta. Indian Council of Agricultural Research. New Delhi.

Drew, S.W. (1981) "Liquid Culture" In manual of Methods of General Bacteriology. Ed. by Costilow, R.N. Am. Soc. of Microbiol. Wash. D.C. pp. 151-178.

Drews, G. & Weckesser, J. (1982) Function, structure and composition of cell walls and external layers. In The Biology of Cyanobacteria Ed. Carr, N.G. & Whitton, B.A. Blackwell Scientific Publications, Oxford Chap 13.

Dreywood, R. (1946) Ind. Eng. Chem. Anal. Ed. 18:499.

Drouet, F. (1951) Cyanophyta. In Smith, Manual of Phycology. Chronica Botanica. Waltham Mass. 159-166.

Drouet, F. & Daily, W. (1956) Revision of the Coccoid Myxophyceae. Butler Univ. Bot. Studies. 12:1-218.

Dudman, W.F. (1977) The role of surface polysaccharides in natural environments. In Surface Carbohydrates of the Procaryotic Cell. Ed, Sutherland, I. Acad. Press.

Duguid, J.P. & Wilkinson, J.F. (1953) The influence of culture conditions on polysaccharide production by Aerobacter aerogenes. J. Gen. Microbiol. 9:174-189.

Dunn, J.H. & Wolk, C.P. (1970) Composition of the cellular envelope of Anabaena cylindrica. J. of Bacteriol. 103:153-158.

Echlin, P. & Morris, I. (1965) The relationship between blue-green algae and bacteria. Biol. Rev. 40:143.

Eichhorn, G.L. (1975) Active sites of biological macromolecules and their interaction with heavy metals. In Ecological Toxicology Research. Plenum Press NY Ed. McIntyre, A.D. & Mills, C.F. Chap.6 pp 23.

Elenkin, A.A. (1936) Monographic algarum Cyanophycearum aquidulcium et terrestrium infinibus URSS inventarum, vol I pp 675 Acad. Nauk. URSS Moscow, Leningrad.

Engleman, T.W. (1881) Neue Methode zur Untersuchung der Sauerstoff auschedung pflanzlicher und thierischer Organismen. Pflugers Arch.ges Physiol. 25:285-292.

Fazzio, S.A.; Uhlinger, D.J.; Parker, J.H. & White, D.C. (1982) Estimations of uronic acids as quantitative measures of extracellular and cell wall polysaccharide polymers from environmental samples. Applied & Env. Microbiol. 43:1151-1159.

Findley, D.L.; Walne, P.L. & Holton, R.W. (1970) The effects of light intensity on the ultrastructure of Chlorogloea fritschii, Mitra grown at high temperature. J. Phycol. 6:182-188.

Fogg, G.E. (1952) The production of extracellular nitrogenous substances by a blue-green alga. Proc. R. Soc. B 139:372-397.

Fogg, G.E. (1971) Extracellular products of algae in freshwater. Arch. Hydrobiol. Beih. Ergebn. Limnol. 5:1-25.

Fremy, P. (1934) Les Cyanophyceae des cotes d'Europe. Mem. Soc. nat. Sci. nat. et Math. de Cherbourg. 41:1-236.

Fritsch, F.E. (1945) The interrelations and class of the Myxophyceae (Cyanophyceae). New Phytol. 41:134-148

Gadd, G.M. & Griffiths, A.J. (1978) Microorganisms and heavy metal toxicity. Microbial Ecology. 4:303-317.

Gallon, J.R.; LaRue, T.A. & Kurz, W.G.W. (1972) Characteristics of nitrogenase activity in broken cell preparations of the blue green alga Gloeocapsa LB 795. Can J. Microbiol. 18:327-332.

Gallon, J.R.; Kurz, W.G.W. & LaRue, T.A. (1975) The physiology of nitrogen fixation by a Gloeocapsa species. In Nitrogen Fixation by Free-living Microorganisms. Ed. Stewart, W.D.P. Cambridge Univ. Press, Cambridge pp. 159-173.

Gallon, J.R. (1980) Nitrogen fixation by photoautotrophs. In Nitrogen Fixation. Ed. Stewart, W.D.P. & Gallon, J.R. Acad. Press London pp. 197-237.

Geesey, G.G. (1982) Microbial exopolymers: ecological & economic considerations. ASM News 48:9-14.

Geitler, L. (1942) Schizophyceae in Engler-Prantl. Die Naturlichen Pflanzenfamilien IInd. Ed., Ia.

Gerhardt, P. (1981) Ed. of Manual of Methods for General Bacteriology. American Society for Microbiology. Wash D.C. Chap. 17.6.6.

Gibson, C.E. (1972) The algicidal effect of copper on a green and a blue green alga and some ecological implications. J. Apllied Ecology. 9:513-518.

Golecki, J.R. & Drews, G. (1974) Cytobiologie 8:213:227.

Griffiths, A.J. (1984) A descriptive numericlature for isolates of cyanobacteria. Br. Phycol. J. 19:233-238.

Grover, I.S. & Puri, S. (1978) Nitrogen fixation by Gloeocapsa. Phykos. 18:125-127.

Harvey, R.S. & Patrick, R. (1967) Concentration of ^{137}Cs, ^{65}Zn and ^{85}Sr by freshwater algae. Biotech. Bioeng. 9:449-456.

Haug, A. & Larsen, B. (1971) Carb. Res. 17:297.

Haug, A.; Larsen, B. & Smidsrod, O. (1974) Ur nic acid sequence in alginate from different sources. Carb. Res. 32:217-225.

Herbst, V. & Overbeck, J. (1978) Metabolic coupling between the alga O. redekei and accompanying bacteria. Naturwissenschaften 65:598-599.

Hestrin, S. (1949) The reaction acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. 180:249-261.

Hough, L.; Jones, J.K.N. & Wadman, W.L.T. (1952) An investigation of the polysaccharide components of certain freshwater algae. J. Chem. Soc. 3393-3399.

Hughes, E.D.; Gorhem, P.R. & Zehnder, A. (1958) Toxicity of a unialgal culture of Microcystis aeruginosa. Can. J. Microbiol, 4:225-236.

Ikeda, F.; Shuto, H.; Saito, T.;Fukui, T. & Tomita, K. (1982) An extracellular polysaccharide produced by Zoogloea ramigera 115. Eur. J. Biochem. 123:437-445.

Jones, T.M. & Albersheim, P. (1972) A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. Plant Physiol. 49:926-936.

Jurgens, U.J. & Weckesser, J. (1985) The fine structure and chemical composition of the cell wall and sheath layers of cyanobacteria. Ann. Inst. Pasteur Microbiol. 136A:41-44.

Kallas, T.; Rippka, R.; Coursin, T. Rebrene, M.C.; DeMarsac, N.T. & Cohen-Bazire, G. (1983) Aerobic nitrogen fixation by non-heterocystous cyanobacteria. In Photosynthetic Procaryotes: Cell Differentiation and Function. Ed. Papageorfiou, G.C. & Packer, L. Elsevier Biomedical NY pp.281-302.

Kessel, M. & Eloff, J.N. (1975) The ultrastructure and development of the colonial sheath of Microcystis marginata. Arch. Microbiol. 106:209-214.

Kirscher, O. (1900) Schizophyceae in Engler and Pranlyl, Die Naturlichen Pflanzenfam., I, 1a, 45-92.

Klein, G. (1915) Zur Chemie der Zellhaut der Cyanophycean. Sitzb. Akad. wiss. Wein, Mat. nat. Kl. I 124:529-545.

Kokyrsta, P.N. & Chekoi, V.N. (1972) Ser. Biol. Khim. (Russian) 2:46-48.

Konopka, A. & Schnur, M. (1980) Effect of light intensity on macromolecular synthesis in cyanobacteria. Microbiol. Ecology 6:291-301.

Kuentzel, L.E. (1969) Bacteria, carbon dioxide and algal blooms. J. Water Pollut. Control Fed. 41:1737-1747.

Lammont, H.C. (1969) Shear-oriented microfibrils in the mucilagenous investments of two motile Oscillatorian blue-green algae. J. Bact. 97:350-361.

Lange, W. (1967) Effects of carbohydrates on the symbiotic growth of the planktonic blue green algae with bacteria. Nature, London 215:1277-1278.

Lange, W. (1976) Speculations on a possible essential function of the gelatinous sheath of blue green algae. Can.J. Microbiol. 22:1181-1185.

Laube, V.M.; McKenzie, C.N. & Kushner, D.J. (1980) Strategies of response to copper, cadmium and lead by a blue green and a green alga. Can. J. Microbiol. 26:1300-1311.

Leak, L.V. (1967) Fine structure of the mucilagenous sheath of Anabaena sp. J. Ultrstructure Res. 21:61-74.

Lehrfeld, J. (1985) Simultaneous gas-liquid chromatographic determination of aldonic acids and aldoses. Anal. Chem. 57:346-348.

Leppard, G.G; Massalski, A. & Lean, D.R.S. (1977) Electron-opaque microscopic fibrils in lakes: Their demonstration, their biological derivation and their potential significance in the redistribution of cations. Protoplasma 92:289-309.

Lessie, T.G. & Whiteley, H.R. (1969) Properties of threonine deaminase from a bacterium able to use threonine as sole source of carbon. J. of Bact. 100:878-889.

Les, A & Walker, R.W. (1983) Toxicity and binding of copper, zinc and cadmium by the blue green alga, Chroococcus paris. Water, Air & Soil Poll. 23:129-139.

Lindberg, B.; Lonngren, J. & Svensson, S. (1975) Specific degradation of polysaccharides. Adv. Carb. Chem. Biochem. 241:3845-3851.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. & Randall, R.J. (1951) Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Ma, T.S. & Rittner, R.C. (1979) Modern Organic Elemental Analysis. Marcel Dekker Inc. New York, NY.

Martin, T.C. Wyatt, J.T. (1974) Extracellular investments in blue green algae with particular empasis on the genus Nostoc. J. Phycol. 10:204-210.

McCandles, (1981) The Biology of the Seaweeds. Bot. Monographs. Ed. Lobban, C. & Wynne, M.J. Univ. of Calif. Press, Berkeley & Los Angeles. Vol. 17.

McKnight, D.M. & Morrel, F.M.M. (1979) Release of weak and strong copper-complexing agents by algae. Limnol. Oceanogr. 24:823-837.

Mehta, V.B. & Vaidya, B.S. (1978) Cellular and extracellular polysaccharides of the blue green alga Nostoc. J. of Exp. Bot. 29:1423-1430.

Millineaux, P.M.; Gallon, J.R. & Chapin, A.E. (1981) Acet lene reduction (nitrogen fixation) by cyanobacteria grown under alternating light-dark cycles. FEMS Microbiol. Lett. 10:245-247.

Moore, B.G. & Tischer, R.G. (1964) Extracellular polysaccharides of algae: Effects on life-support systems. Science 145:586-587.

Moore, B.G. & Tischer, R.G. (1965) Biosynthesis of extracellular polysaccharides by the blue green algae A, flosaquae. Can.J. Microbiol. 11:877-885.

Morris, E.R.; Rees, D.A.; Robinson, G. & Young, G.A. (1980) Competitive inhibition of interchain interactions in polysaccharide systems. J. Mol. Biol. 138:363-374.

Naegeli, (1849) Gattungen einzelliger algen, Physiologisch und Systematisch bearbeitet. Neue Denkschriften Allg. Schweiz. Natur. Ges. 10:139. Zurich

Overbeck, J. (1975) Distribution pattern of uptake kinetic responses in a stratified eutrophic lake. Verh. int. Verein theor. angew. Limnol. 19:2600-2615.

Paerl, H.W. (1978) Light-mediated recovery of nitrogen fixation in the blue green algae Anabaena sp. in oxygen supersaturated waters. Oecologia (Berlin) 32:135-139.

Paerl, H.W. (1979) Microbial organic carbon recovery in aquatic ecosystems. Limnol. Oceanogr. 23:927-935.

Paerl, H.W. & Bland, P.T. (1982) Localized tetrazolium reduction in relation to nitrogen fixation, carbon dioxide fixation and hydrogen uptake in aquatic filamentous cyanobacteria. Appl. Env. Microbiol. 43:218-226.

Paerl, H.W. & Keller, P.E.(1978) Significance of bacterial-Anabaena (cyanophyceae) associations with respect to nitrogen fixation in freshwater. J. Phycol. 14:254-260.

Painter, T.J. (1983) Algal Polysaccharides. In The Polysaccharides Ed. Aspinall, G.O. Academic Press NY Vol. 2 Chap. 4 pp 206.

Papenfuss, G,F. (1955) Class of the algae in a century of progress in natural sciences 1893-1953. Calif. Acad. Sci. San Fransisco 115-224.

Pate, J.L. & Ordal, J.E. (1967) J. of Cell Biology. 35:37-44.

Patrick, F.M. & Loutit, M. (1976) Passage of metals in effluents through bacteria to higher organisms. Water Res. 10:333-335.

Rees, D.A. (1963) Biochem. J. 88:343.

Rees, D.A. (1972) Polysaccharide gels: a molecular view. Chem Ind. 19:630-636.

Rees, D.A. (1975) Stereochemistry and binding behavior of carbohydrate chains. MTP Int. Rev. of Sci. Biochem. series 1 Univ. Park Press Ed. Whelan W.J. vol. 5 chap. 1.

Rees, D.A. (1977) Polysacchraide Shapes. John Wiley & Sons. NY. pp80.

Ridgeway, H.F.; Means, E.G. & Olson, B.H. (1981) Iron bacteria in drinking-water distribution systems: elemental analysis of Gallionella stalks using X-ray energy dispersive microanalysis. Appl. Env. Micro. 41:288-297.

Rippka, R.; Neilson, A.; Kunisawa, R. & Cohen-Bazire, G. (1971) Nitrogen fixation by unicellular blue green algae. Arch Mikrobiol. 76:341-348.

Rippka, R.; Deruelles, J.; Waterbury, J.B.; Herdman, M. & Stanier, R. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. of Gen. Microbiol. 111:1-61.

Rippka, R. & Herdman, M. (1985) Division patterns and cellular differentiation in cyanobacteria. Ann. Inst. Pasteur Microbiol. 136A: 33-39

Rodrigues, K. (1986) The effect of cadmium upon the growth and nitrogen fixation of the cyanobacterium Gloeothece ATCC 27152. Masters Thesis, University of Massachusetts.

Rosen, H. (1957) A modified ninhydrin colorimetric analysis for amino acids. Arch of Biochem & Biophysics 67:10-15.

Roth, I.L. (1977) Physical structure of surface carbohydrates. In Surface Carbohydrates of the Procaryotic Cell. Ed. Sutherland, I.W. Acad. Press London-NY-San Francisco. Chap 2.

Sangar, V.K. & Dugan, P.R. (1972) Polysaccharide produced by Anacystis nidulans: Its ecological implications. Appl. Microbiol. 24: 732-734.

Scherrer, R. & Gerhardt, P. (1971) Molecular sieving by the Bacillus megaterium cell wall and protoplast. J. of Bact. 107:718-735.

Schrader, M.; Drews, G.; Golecki, J.R. & Weckesser, J. (1982) Isolation and characterization of the sheath from the cyanobacterium Chlorogloeopsis PCC 6912. J. Gen Microbiol. 128:267-272.

Shilo, M. (1970) Lysis of blue green algae by Myxobacter. J. of Bact. 104:453-461.

Singh, P.K. (1973) Nitrogen fixation by the unicellular blue green alga Aphanothece. Archiv. fur Mikrobiologia. 92:59-62.

Sloeneker, J.H. & Jeanes, A. (1962) Exocellular bacterial polysaccharide from Xanthomonas campestris NRRL B-1459. Can. J. Chem. 40:2066-2071.

Smith, G.M. (1950) The Freshwater Algae of the United States. second edition McGraw Hill NY-Toronto-London

Smith, H. (1977) Bact. Rev. 41:475-500.

Standard Methods for the Examination of Water and Wastewater. Am. Pub. Health Assoc. Wash. D.C. 15 Ed.

Stanier, R.Y.; Kunisawa, R.; Mandel, M. & Cohen-Bazire, G. (1971) Purification and properties of unicellular blue green algae (order Chroococcales). Bact. Revs. 35:171-205.

Stanier, R.Y. & Cohen-Bazire, G. (1977) Phototrophic procaryotes: the cyanobacteria. Ann. Rev. Microbiol. 31:225-274.

Starr, R.C. (1964) Culture collection of algae at Indiana University Division Cyanophyta. Am. J. of Bot. 51:1034-1035.

Tischer, R.G. & Davis, E.B. (1971) The effect of various nitrogen sources upon the production of extracellular polysaccharide by the blue green alga Anabaena flos-aquae A37. J. of Exp. Bot. 22:546-551.

Tischer, R.G. & Moore, B.G. (1964) An extracellular polysaccharide produced by Palmella mucosa Kutz. Archiv. fur Mikrobiologie. 49:158-166.

Tonn, S.J. & Gander J.E. (1979) Biosynthesis of polysaccharides by procaryotes. Ann. Rev. Microbiol. 33:169-199.

Troy, F.A. (1979) The chemical and biosynthesis of selected bacterial capsular polymers. Ann. Rev. Microbiol. 33:519-560.

Tuffery, A.A. (1969) Light and electron microscopy of the sheath of a blue green alga. J. Gen. Microbiol. 57:41-50.

Uhlinger, & White (1983) Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in Pseudomonas atlantica. Appl. Env. Microbiol. 45:64-70.

Vaara, T. (1982) The outermost surface structure of chroococcacean cyanobacteria. Can. J. Microbiol. 28:929-941.

Vadas, L.; Prihor, H.S.; Pugashetti, B.K. & Feingold, D.S. (1981) A gas chromatographic method for the quantitative determination of hexuronic acids in alginic acid. Anal. Biochem. 114:294-298.

Van Den Berg, C.M.G; Wong, P.T.S. & Chau, Y.K. (1979) Measurement of complexing materials excreted from algae and their ability to ameliorate copper toxicity. J. Fish. Res. Board. Can. 36:901-905.

Wang, W.S. & Tischer, R.G. (1973) Study of the extracellular polysaccharides produced by a blue green alga, Anabaena flos-aquae A37. Arch. Mikrobiol. 91:77-81.

West, G.S. & Fritsch, F.E. (1927) A treatise on the British freshwater algae. Univ. Press, Cambridge.

Wetzel, R.G. (1975) Limnology. Saunders Philadelphia pp306.

Wilkinson, J.F.; Duguid, J.P. & Edmunds, P.N. (1954) The distribution of polysaccharide in Aerobacter and Escherichia strains and its relation to antigenic character. J. Gen. Microbiol. 11: 59-72.

Wolle, F. (1887) Freshwater Algae of the Unted States. The Comenius Press, Bethlehem, PA.

Wyatt, J.T. & Silvey, J.K.G. (1969) Nitrogen fixation by Gloeocapsa. Science 165:908-909.

Zevenhuizen, L.P.T.M. & Ebbink, A.G. (1974) Exocellular pyruvate-containing galactoglucan of Archromobacter spp. Arch. Microbiol. 96:75-82.

Zobell, C.E. (1943) The effect of solid surfaces upon bacterial activity. J. Bact. 46:39-56.

Zobell, C.E. & Anderson, D.O. (1936) Observations on multiplication of bacteria in different volumes of stored seawater and the influence of oxygen tension and solid surfaces. Biol. Bull. (Woods Hole, Mass.) 71:324-332.